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PROTON EXCHANGE BEHAVIOUR OF PROTEINS

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PROEFSCHRIFT

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"En al klinkt dat soms wat raar,
wat gedrukt staat, dat is waar".

Uit: Reizen en avonturen van mijnheer Prikkebeen.

CONTENTS

GENERAL INTRODUCTION

This study was originally focused on the problem whether it would be possible to measure the conductivity at zero ionic strength of isoionic protein solutions due to proton charge fluctuations. This conductivity was expected by van Os, Möller and Overbeek[1-3] and a relationship was given by them between this conductivity and the standard deviation S of these proton charge fluctuations. Some indication of the existence of the phenomenon had also been found by them, measuring the electric transference of alkali albuminates. After this problem had been solved successfully, the idea was to measure the quantity S also by using an independent way of determination, viz. from the slope of the hydrogen ion titration curve at the isoionic point at zero ionic strength. An accurate determination, however, of the differential titration curve -in which this slope is plotted vs. the mean proton charge of the protein- had not yet been performed. Therefore we had to refine the normally used titration techniques in such a manner that this differential titration curve could be obtained. In treating this problem we entered into an area of investigation correlated to the study of the charge fluctuations, viz. the study of the proton exchange of proteins in relationship to their structural properties. This step was a logical consequence because it appeared that the study of the latter subject was much facilitated by analysing the differential titration curve instead of the normal titration curve. These are, shortly, the arguments which led to the investigations presented in this thesis. Most of the work has already been published in a set of four papers[4-7].

As both areas of investigations are grounded on the theory of the multiple equilibria the main features of this theory are given in chapter 1.
In chapter 2 the technique of the determination of differential titration curves is described and some examples of the interpretation of such curves are given.In chapter 3 a study of the Bohreffect of hemoglobin by means of differential titration curves is described, while chapter 4 deals with the charge fluctuations of a number of proteins.

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Chapter 1.

MULTIPLE EQUILIBRIA

1.1 General theory [1-4].

When a macromolecule P has n sites which combine reversibly with an ion or molecule B, then the system can be described by the following set of equilibria

$$
\begin{array}{lcll}
P_o & + B \rightleftharpoons & PB \\
\vdots & \vdots & \vdots \\
PB & + B \rightleftharpoons & PB_2 \\
\vdots & \vdots & \vdots \\
PB_{\nu-1} & + B \rightleftharpoons & PB_\nu \\
\vdots & \vdots & \vdots \\
PB_{n-1} & + B \rightleftharpoons & PB_n
\end{array}
$$

P_o and PB_n are the states of zero and maximum binding respectively and PB_ν the state in which the macromolecule has ν particles bound. The probability $w(\nu)$ of finding the molecule in the state characterized by the formula PB_ν is given by

$$w(\nu) = \frac{(PB_\nu)}{\sum_{\nu=o}^{n} (PB_\nu)} \qquad (1.1.1)$$

in which (PB_ν) is the concentration of all molecules having ν particles bound an $\sum_{\nu=o}^{n} (PB_\nu)$ is the total concentration of the macromolecule.

The equilibrium constant K_ν of the overall reaction $P_o + \nu B \rightleftharpoons PB_\nu$ is

$$K_\nu = \frac{(PB_\nu)}{(P_o)A^\nu} \cdot \frac{f_\nu}{f_o}$$

where A represents the activity of free B, f_ν the activity coefficient of species PB_ν and f_o that of P_o. Eqn. (1.1.1) can so be written as

$$w(\nu) = \frac{K_\nu A^\nu f_\nu^{-1}}{\sum_{\nu=o}^{n} K_\nu A^\nu f_\nu^{-1}} \qquad (1.1.2)$$

When ions are bound f_ν can be written, at least in first approximation, as $f_\nu = f_c . \gamma_\nu$,where f_c represents the non electrostatic part of f_ν which is supposed to be independent of ν , and γ_ν , the electrostatic part which will be strongly dependent on ν . f_c, being a constant, cancels in eqn. (1.1.2).
So the expression for $w(\nu)$ becomes

$$w(\nu) = \frac{K_\nu A^\nu \gamma_\nu^{-1}}{\sum_{\nu=o}^{n} K_\nu A^\nu \gamma_\nu^{-1}} \qquad (1.1.3)$$

In section 1.4 it will be shown that using a proper model a useful expression for γ_ν can be derived.
In binding experiments the only observable quantities are A and the average number of bound particles $\bar{\nu}$; $\bar{\nu}$ is given by[5]

$$\bar{\nu} = \sum_{\nu=o}^{n} \nu . w(\nu) = \frac{\sum_{\nu=o}^{n} \nu K_\nu A^\nu \gamma_\nu^{-1}}{\sum_{\nu=o}^{n} K_\nu A^\nu \gamma_\nu^{-1}} \qquad (1.1.4)$$

For the power series in the denominator of eqn. (1.1.4)

$$Y = \sum_{\nu=o}^{n} K_\nu A^\nu \gamma_\nu^{-1} \qquad (1.1.5)$$

We have $$A \frac{dY}{dA} = \sum_{\nu=0}^{n} \nu K_\nu A^\nu \gamma_\nu^{-1}$$

(and in general

$$A^q \frac{d^q Y}{dA^q} = \sum_{\nu=0}^{n} \frac{\nu!}{(\nu-q)!} K_\nu A^\nu \gamma_\nu^{-1}) \qquad (1.1.6)$$

With this relation eqn. (1.1.4) becomes

$$\bar{\nu} = \frac{d \ln Y}{d \ln A} \qquad (1.1.7)$$

It will be clear that, if only $\bar{\nu}$ and A are known, it will hardly be possible to find all constants K_ν. Only, when the sites can be divided into several classes of identical groups, it is possible to simplify Y in such a way that from eqn. (1.1.7) a useful relation can be obtained, which enables us to interpret experimental observations in terms of molecular parameters and thermodynamic quantities. It must be noted that in deriving eqn. (1.1.7) we have implicitly assumed that γ_ν and A are independent of each other. In general this will not be true. In many cases, however, the experimental conditions can be chosen in such a way that the ionic strength and so γ_ν - see further - is kept constant when the activity A is changed.

1.2 Calculation of the average number of particles bound in the case of n identical and independent sites.

In this hypothetical situation corresponding with the supposition that the dielectric constant D of the medium is infinite all activity coefficients are unity. So we may write for the concentration (PB_ν) of all molecules with ν particles bound

$$(PB_\nu) = K_\nu C^\nu (P_o) \qquad (1.2.1)$$

in which C is the concentration of free B.
The molecules, however, making up the total concentration (PB_ν)

differ in the way by which ν particles are distributed among then available sites. Because all sites are supposed to be identical and independent, the number of possible distributions or configurations is equal to

$$\binom{n}{\nu} = \frac{n!}{\nu!(n-\nu)!} \qquad (1.2.2)$$

Indicating one of the possible configurations by PB^*_ν and its concentration by (PB^*_ν) we can write for (PB_ν)

$$(PB_\nu) = \frac{n!}{\nu!(n-\nu)!}\,(PB^*_\nu) \qquad (1.2.3)$$

(PB^*_ν) is related to (P_o) by the equilibrium

$$P_o + \nu B \rightleftharpoons PB^*_\nu$$

so that

$$(PB^*_\nu) = K_\nu C^\nu (P_o) \qquad (1.2.4)$$

Substituting eqn. (1.2.4) into eqn. (1.2.3) gives

$$(PB_\nu) = \frac{n!}{\nu!(n-\nu)!}\,K^*_\nu C^\nu (P_o) \qquad (1.2.5)$$

From the eqns. (1.2.5) and (1.2.1.) it follows:

$$K_\nu = \frac{n!}{\nu!(n-\nu)!}\,K^*_\nu \qquad (1.2.6)$$

The species PB^*_ν with a fixed configuration can be built up as follows

$$\begin{array}{lcll} P_o & + & B & \rightleftharpoons & PB^* \\ \vdots & & \vdots & & \vdots \\ PB^* & + & B & \rightleftharpoons & PB^*_2 \\ \vdots & & \vdots & & \vdots \\ PB^*_{\nu-1} & + & B & \rightleftharpoons & PB^*_\nu \end{array}$$

Because the sites are supposed to be identical and independent, the change in free energy of each step will be equal; so the binding of each particle B is ruled by the same intrinsic equilibrium constant K.

Hence we have

$$(PB^*) = K.C.(P_o)$$

$$(PB_2^*) = K.C.(PB^*) = K^2C^2(P_o)$$

$$(PB_\nu^*) = K.C.(PB_{\nu-1}^*) = K^\nu C^\nu (P_o) \qquad (1.2.7)$$

Combining the eqns. (1.2.4) and (1.2.7) we can write

$$K_\nu^* = K^\nu \qquad (1.2.8)$$

Substituting this expression for K_ν^* in eqn. (1.2.6) we get

$$K_\nu = \frac{n!}{\nu!(n-\nu)!} K^\nu \qquad (1.2.9)$$

For eqn. (1.1.5) we can write

$$Y = \sum_{\nu=o}^{n} K^\nu C^\nu \qquad (1.2.10)$$

Substitution of eqn. (1.2.9) yields

$$Y = \sum_{\nu=o}^{n} \frac{n!}{\nu!(n-\nu)} K^\nu C^\nu \qquad (1.2.11)$$

The right-hand side of this relation is the binomial expansion[5] of

$$Y = (1 + K.C.)^n \qquad (1.2.12)$$

Substituting this expression for Y in eqn. (1.1.7) gives the simple relation

$$\bar{\nu} = \frac{n\ K\ C}{1 + K\ C} \qquad (1.2.13)$$

This relation can be written as[6]

$$\frac{\bar{\nu}}{C} = nK = \bar{\nu}\ K \qquad (1.2.14)$$

By plotting $\frac{\bar{\nu}}{C}$ vs $\bar{\nu}$, K is obtained from the slope of the curve and n from the intercept at $\frac{\bar{\nu}}{C} = 0$.

1.3 Calculation of the average number of particles bound if there are i classes with n_i identical and independent sites.

In most cases the supposition that all sites are identical is to simple. Mostly, however, the total number of sites can be divided into i different classes of identical sites.

In the same way as in the preceding section it can be derived that

$$\bar{\nu}_i = \frac{n_i K_i C}{1+K_i C} \qquad (1.3.1)$$

in which n_i is the number of sites of class i, K_i is the corresponding intrinsic equilibrium constant, and $\bar{\nu}_i$ the average number of particles bound by the sites of class i. The expression for $\bar{\nu}$, the mean value of particles bound by all classes, now becomes

$$\bar{\nu} = \sum_i \bar{\nu}_i = \sum_i \frac{n_i K_i C}{1+K_i C} \qquad (1.3.2)$$

With $\Theta_i = \frac{\bar{\nu}_i}{n_i}$ representing the fraction of n_i having reacted, eqn. (1.3.2) can be written as

$$\bar{\nu} = \sum_i n_i \Theta_i \qquad (1.3.3)$$

The presence of more than one class emerges directly from the plot of $\frac{\bar{\nu}}{C}$ vs. $\bar{\nu}$. Instead of a linear relationship between $\frac{\bar{\nu}}{C}$ and $\bar{\nu}$ a more hyperbolic curve will be obtained.

In many cases there will not be any information about the number of the several classes and the nature of the binding sites; to evaluate each term of eqn. (1.3.2) is then a matter of trial and error and the solution obtained will not be unique. In other cases, however, especially in studying the proton exchange behaviour of macromolecules some information about the number of classes, the values of n_i and K_i is available.. This is of great help in analysing the hydrogen ion equilibria of these macromolecules. This information -for proteins for example the amino acid analysis- is however approximate and is in most cases not more than a helpful guide.

1.4 Calculation of the electrostatic part of the activity coefficient of macromolecules, when the sites are identical, but not independent.

The assumption made in the preceding sections, viz. that the groups will associate with particles independently of each other, is unrealistic. Especially, when the bound particles are ions - to this case we will restrict our attention - it will be obvious that because of electrostatic interactions the binding at a certain site will influence the binding at all other sites and vice versa. At each site i a potential $\phi_i(\nu)$ will be generated by the other charged sites. $\phi_i(\nu)$ will be different for each site and dependent on ν.
So the standard free enthalpy change $\Delta F_i^o(\nu)$ of binding at site i will be equal to (see for instance ref. 7)

$$\Delta F_i^o(\nu) = \Delta F_{int,i}^o + z_B e \varphi_i(\nu) \qquad (1.4.1)$$

in which $\Delta F_{int,i}^o$ is the standard free enthalpy change when all sites (groups) are moving freely through the solution -with retention of nonelectrostatic conformationally induced influences on $\Delta F_{int,i}^o$-, z_B the valence of the ion, sign included, and e the elementary unit of charge*.

Calculation of $\phi_i(\nu)$ requires that the relative positions of all groups are known precisely at each value of ν, together with the state of charge of all sites. This information, however, always is lacking and we have to use approximate solutions.
The study of the binding of ions by protein molecules -to which our

* Note:
So far the approach of the problem is analoguous to the treatment given in ref. 7 for polyelectrolyte solutions in general. The elaboration of eqn. (1.4.1) for a protein molecule gives the same result as the way of calculation of the electrostatic energy correction for proteins as presented in the commonly used handbooks[1-4]. The treatment presented here differs somewhat from that being found in these books in the way of looking to the problem. It must be stressed, however, that the difference is not essential.

attention will further be restricted- is favoured by the fact that over a great range of $\bar{\nu}$ the sites are located on the surface of a compact impenetrable macromolecule. Under these conditions it is possible to obtain an appropriate expression for $\phi_i(\nu)$ by assuming that all charges on the surface are randomly distributed and that the protein can be represented by a sphere. The approach to the problem is equivalent to the assumption that the protein can be represented by a conducting sphere with a net charge equal to the sum of the charges being present; it is also equivalent to neglecting the contribution of electric dipoles and higher multipoles to the electric field.

In this model the potential $\phi_i(\nu)$ is equal at all sites -consequently the subscript i in eqn. (1.4.1) can be omitted- and is given by[1]

$$\varphi(\nu) = \frac{Ze}{DR}\left(1 - \frac{xR}{1+xa}\right) \qquad (1.4.2)$$

where Ze = the total charge of the protein, D = dielectric constant of water, R = radius of the protein, x = Debye-Hückel parameter and a = the distance of closest approach between the protein and the counterions. The factor within brackets is the so called Debye-Hückel screening factor. The potential $\frac{Ze}{DR}$ of the protein is screened because of the space charge built up by the counterions present in the solution. With $\Delta F = -kT \ln K$ and $\Delta F_{int} = -kT \ln K_{int}$ eqn. (1.4.1) becomes

(1.4.3)

$$-KT \ln K = -KT \ln K_{int} + \frac{z_B Ze^2}{DR}\left(1 - \frac{xR}{1+xa}\right)$$

or

$$K = K_{int}\, e^{-2wz_BZ} \qquad (1.4.4)$$

with

$$w = \frac{e^2}{2DRkT}\left(1 - \frac{xR}{1+xa}\right) \qquad (1.4.5)$$

w is called the electrostatic interaction factor.

Substituting eqn. (1.4.4) into eqn. (1.2.9) gives

$$K_\nu = \frac{n!}{\nu!(n-\nu)!} K_{int}^{\nu} \; e^{-2w \; \nu \; z_B Z} \qquad (1.4.6)$$

or

$$K_\nu = K_\nu' \; e^{-2w \; \nu \; z_B Z} . \; A^\nu \qquad (1.4.7)$$

in which K_ν' is the value of K_ν, when Z = 0, or in other words, when there are no electrostatic interactions.
Substitution of eqn. (1.4.7) into eqn. (1.2.10) and replacing C, the concentration of free B, by its activity A yields

$$Y = \sum_{\nu=o}^{n} K_\nu' \; e^{-2w \; \nu \; z_B Z} . \; A^\nu$$

Combining this equation with the general expression for Y as given by eqn. (1.1.5) we can write for γ_ν

$$\gamma_\nu = e^{2w \; \nu \; z_B Z} \qquad (1.4.8)^*$$

Combining eqn. (1.4.7) with eqn. (1.2.11) gives, replacing C by A

$$Y = \sum_{\nu=o}^{n} \frac{n!}{\nu!(n-\nu)} (K_{int} . \; e^{-2wz_B Z})^\nu A^\nu \qquad (1.4.9)$$

from which it follows by applying eqn. (1.1.7) and generalising for several classes of sites

$$\bar{\nu} = \sum_i \frac{n_i \; K_{int,i} \; e^{-2wz_B Z} . \; A}{1 + K_{int,i} \; e^{-2wz_B Z} . \; A} \qquad (1.4.10)$$

* Note:
It should be noted that the same expression for γ_ν would be obtained even if all sites would be non-identical. This becomes clear if one takes in mind that the extra work required $z_B e \; \phi \; (\nu)$ will be independent from the value of the K_{int} of a site.

Often the binding of ions is described as a dissociation proces. When $\bar{r}$ is the number of ions given of by the protein ($\bar{r} = n - \bar{\nu}$) and $K_{int,i}$ is now read as the dissociation constant, r is given by

$$\bar{r} = \sum_i \frac{n_i K_{int,i} e^{2wz_B Z/A}}{1 + K_{int,i} e^{2wz_B Z/A}} = \sum_i n_i \bar{r}_i \qquad (1.4.11)$$

or

$$\bar{r} = \sum_i n_i \alpha_i \qquad (1.4.12)$$

in which α_i represents the dissociated fraction of n_i. The model described appears to work quite well, especially the ionic strength dependence as given by eqn. (1.4.5) is mostly in good agreement with the observation. The electrostatic energy correction w, however, is often found smaller than calculated according to eqn. (1.4.5).

1.5 The standard deviation of the number of bound particles.

Because the value of $K_\nu / K_{\nu-1}$ is finite, the actual number of bound ions ν will fluctuate, in course of time, around the mean value $\bar{\nu}$. The spread of $w(\nu)$ about $\bar{\nu}$ is given by the standard deviation S of $w(\nu)$ and is defined as

$$S^2 = \sum_{\nu=0}^{n} (\nu - \bar{\nu})^2 w(\nu) = \overline{\nu^2} - \bar{\nu}^2 \qquad (1.5.1)$$

By means of eqn. (1.1.6) it can easily be shown that

$$S^2 = \frac{d^2 \ln y}{d(\ln A)^2} = \frac{d\,\bar{\nu}}{d \ln A} \qquad (1.5.2)$$

So, S^2 can be determined from the slope of the curve of $\bar{\nu}$ vs. ln A. Eqn. (1.5.2) is known as the Linderstrom-Lang equation (see ref. 4). In the case of i classes of identical and independent sites S^2 is given by

$$S^2 = \sum_i \frac{n_i K_i C}{(1+K_i C)^2} \qquad (1.5.3)$$

or

$$S^2 = \sum_i n_i \Theta_i (1 - \Theta_i) \qquad (1.5.4)$$

As a result of this distribution of ν around $\bar{\nu}$, the charge Z will also fluctuate around the mean value $\bar{Z}$. In chapter 4 it will be shown that, when $\bar{Z} = 0$, this will have important physical consequences.

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Chapter 2

DETERMINATION AND INTERPRETATION OF THE DIFFERENTIAL TITRATION CURVES OF PROTEINS

2.1 Introduction

A protein possesses several classes of groups which can exchange protons with the solvent. This exchange can be studied by means of the hydrogen ion titration curves, in which the number of protons given off by the protein is plotted vs. the pH. The study of such titration curves has proved to be a powerful tool in the determination of the number of groups of the several classes and their thermodynamic properties. Moreover, information about structural properties can be obtained. Reviews on the subject have been given by Tanford[1-3], Steinhardt and Beychok[4], and Edsall and Wyman[5]. Usually, a hydrogen ion titration curve of a protein is divided into three regions. First the carboxyl region between about pH = 2 and pH = 5.5, next a neutral region from pH = 5.5 to pH = 9.0, containing the imidazole groups and α-amino groups, and finally a basic region above pH = 9.0 where all other groups are titrated. This division into three regions is based upon a rather rough estimation of the inflection points at about pH = 5.5 and pH = 9.0, which are considered as the titration endpoints of the first two regions. From these endpoints the numbers of titratable groups in the acid and neutral region are then approximately derived after which by means of a refining procedure described by Tanford[2] the real numbers can be obtained.It will be clear that a sufficiently accurate determination of the differential titration curve will result in a more precise localisation of the inflection points. This facilitates the normally used analysing procedure. Moreover, an analysis of the differential curve itself, proposed by us, can then be followed, which rapidly leads to the number of titratable groups, their pK-differences, the detection of pH dependent conformational changes etc. Hitherto no method was known by which a precise reproducible determination of the differential titration curve could be made. The first attempt was made by Perlman, Oplatka and Katchalsky[6]. They stated, however, one should not attribute too much significance to the exact shape of the curves because of the poorness of the reproducibility.

In practice, two ways of determining a titration curve are used The first involves mixing of several protein solutions with calculated amounts of acid or base. Each of these solutions corresponds to one point of the curve In the second one, increasing amounts of acid or base are added to the same protein solution. We have tried to improve the latter (continuous) titration to a level, that makes it possible to measure differential titration curves. This attempt could be successful because some modern direct reading pH-meters have such a high stability that the output can be measured by means of a high resolution digital voltmeter with an accuracy, which is about a factor 10 better than the reading accuracy of the moving coil meter of such an instrument It must be stressed that this does not mean that the absolute value of the pH can be measured with higher accuracy, since it is limited by several factors[7], such as precision of standard buffers, liquid junction potentials etc
However, for a differential curve changes in pH must be measured and very small differences can be detected in the way described
By combining the equipment with a microburette of sufficient accuracy good differential curves have been obtained The capabilities of the method will be shown by giving the results for bovine serum albumin

2.2 Analysis of the pH vs Z_H curve

The number of protons given off by a protein molecule is given by eqn. (1.4.11). As reference point for counting r the point of maximum proton binding is taken, at this point all sites are occupied by protons This is normally reached at low pH (pH = 2) because the lowest pK value occuring in proteins is mostly found around pK = 4.0.

In isoionic protein solutions the mean proton charge Z_H of the protein is very close to zero[2], in practice equal to zero Mostly therefore this point of zero net proton charge is chosen as reference point. Z_H is related to $\bar{r}$ by

$$Z_H = Z_m - \bar{r} = Z_m - \sum_i n_i \alpha_i \qquad (2.2.1)$$

where Z_m is the maximum positive charge Z_m is equal to the total number of protons taken up by the molecule with $Z_H = 0$ as reference point and also equal to the sum of all groups getting a positive charge when protons are bound If all groups are able to exchange protons with the solvent, Z_m and each n_i are predicted by the amino acid analysis

Often, however, some groups are in the native structure prevented from taking part in the proton equilibria. These are the so called masked or buried groups. When at low pH a protein looses its native structure the masked groups become titratable[8]. So Z_m observed will then differ from the Z_m that would have been observed, when the protein would have kept its native structure. Therefore we prefer to define Z_m as the sum of all groups becoming positively charged at proton binding provided the protein keeps its native structure.

In studying the proton equilibria of proteins each term r_i of eqn. (1.4.11) is considered separately and written as[1-5]

$$pH = pK_i + \log \frac{\alpha_i}{1-\alpha_i} - 0.868wZ \qquad (2.2.2)$$

in which Z represents the real charge of the protein and $pK_i = pK_{int,i}$ as defined in section 1.4. So there are i of such relations when i is the number of classes. As has been pointed out in chapter 1 Z represents the real charge of the protein which is the sum of the proton charge Z_H and the charge of other bound ions. According to Tanford[2], however, it is reasonable to assume that the number of other bound ions varies approximately linearly with Z_H for most proteins. Indeed, we found, for example, a linear relationship between the number of chloride ions bound by human serum albumin and the proton charge of the protein using the data of Scatchard and Yap[9]. The analysis of the normal titration curve can briefly be summarized as follows[2]: first the titration curve is divided into three regions as is pointed out in the introduction; next one tries to find a correlation between α_i and Z_H in those pH regions in which the α_i of other classes are supposed to be equal to one or to zero; mostly one starts with the carboxyl region.
The next step is to plot $pH - \log \frac{\alpha_i}{1-\alpha_i}$ vs. Z_H. If the slope of this curve remains constant over a broad region of Z_H and if the value of this slope does not differ too much from the value calculated according to eqn. (1.4.5) then the relation between α_i and Z_H is taken as being right. The intercept of the curve at $Z_H = 0$ is then equel to pK_i. Often it occurs that an acceptable value of w cannot be obtained. It might be that w is not a constant or that it deviates much from the calculated value. These features are indications of anomalous behaviour of the protein. When binding of ions other than H^+ ions occurs an apparently

too low value of w is found from the curve of pH - log $\frac{\alpha_i}{1-\alpha_i}$ vs. Z_H. In addition pK_i observed will then be strongly dependent on the concentration of the ions being bound. Finally it must be noticed that in applying eqn. (1.4.5) it is assumed that proteins have a compact spherical structure. For non-spherical molecules the expression for w is more complicated[3], but w_{calc} obtained from the approximate eqn.(1.4.5) usually[3] does not differ more than 10% from w_{calc} obtained by applying the more rigorous expression.

2.3 Analysis of the differential curve.

In the differential titration curve $-\Delta pH/\Delta Z_H$ is plotted vs. Z_H in which Δ pH is the change in pH accompanying the change in proton charge ΔZ_H caused by a small addition of acid or base. To obtain a useful expression for dpH/dZ_H in order to analyse the differential titration curve we proceed as follows. From eqn. (2.2.1) we have

$$dZ_H = - \sum_i n_i \, d\alpha_i \qquad (2.3.1)$$

From equation (2.2.2) it follows

$$dpH = \frac{1}{2.303\,\alpha_i(1-\alpha_i)}\, d\alpha_i - 0.868\,w\,dZ$$

or

$$d\alpha_i = 2.303\,\alpha_i\,(1-\alpha_i)\,(dpH + 0.868\,w\,dZ) \qquad (2.3.2)$$

Combining equations (2.3.1) and (2.3.2) we get (2.3.4)

$$dZ_H = - (dpH + 0.868\,w\,dZ)\; 2.303 \sum_i n_i\alpha_i(1-\alpha_i)$$

When only protons are bound, then $Z \equiv Z_H$ and from eqn. (2.3.4) we find (2.3.5)

$$-\frac{dpH}{dZ_H} = \frac{1}{2.303\,\Sigma_i n_i\alpha_i(1-\alpha_i)} + 0.868\,w$$

When besides protons, other ions also are bound, then $Z = Z_H + Z_b$ (Z_b = charge caused by other bound ions). This leads to an extra term $0.868w\ dZ_b/dZ_H$ in eqn. (2.3.5). This extra term will be a constant when Z_b varies linearly with Z_H. Binding of ions then only means that a constant is added to eqn. (2.3.5). Provided these conditions are fulfilled the differential titration curve will be invariant in shape for changes in ionic strength. This is one of the advantages of the differential titration curve. In the normal titration curve, on the contrary, the pH values at positive and negative Z_H values are shifted in opposite direction when the ionic strength is varied.

As already mentioned in the introduction important points in the titration curve (pH vs. Z_H) of a protein are the inflection points in a transition region where the titration of one class of groups is not yet completed and that of another class has already begun. These points correspond to peaks in the differential curve and our first problem was to find the relation between the position of these peaks, the numbers of groups in the different classes and their pK_i differences. Therefore we calculated the differential titration curves for a number of hypothetical proteins having three different classes of groups with $n_2 = 20$, $n_3 = 60$, $pK_2 = 7$, $pK_3 = 10$ and $Z_m = 80$ for all proteins, but with varying values for n_1 and pK_1, and with $w = 0$. The calculation procedure was as follows : There are in this case three equations of the type of eqn. (2.2.2) by means of which, for example, α_2 and α_3 can be expressed in α_1 (in these expressions w, Z_H and the pH no longer occur). Values for α_1 were chosen and the corresponding values of α_2 and α_3 calculated. Substitution of α_1, α_2 and α_3 in eqn. (2.2.1) gives Z_H, while substitution in equation (2.3.5) gives the corresponding value of dpH/dZ_H. Some of these calculated curves are given in Fig. 1.

As could be expected, the positions of the left and right peak indicates the number of groups in class 1 and 2 with better accuracy the greater ΔpK (= $pK_2 - pK_1$) and the closer the ratio n_1/n_2 approaches unity. When the left peak is pronounced, as in curve A, the error is only a small fraction of one group. So, in this case, an approximate relation $n_c = Z_m - Z_p$ will hold, where Z_p is the value of Z_H at the maximum of the left peak. When this relation, however, is applied to curve C n_1 and n_2 are read one group too high and too low, respectively. In such a case the real numbers of the groups can be found by a method of successive approximation. This is illustrated in Fig. 2. Here curve C from Fig. 1 is

redrawn and considered as "experimental" curve with a known value of Z_m (= 80).

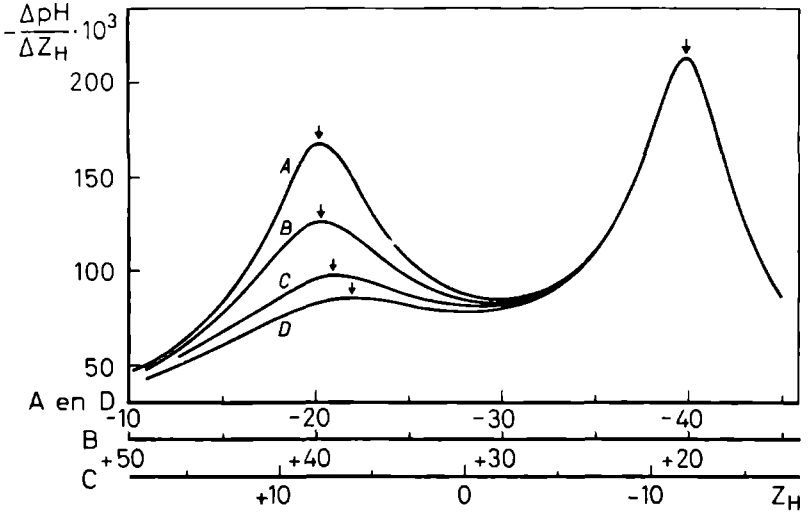

Fig.1. Calculated differential titration curves of some hypothetical proteins, all with 3 classes of groups, viz. carboxyl groups (class 1), imidazole groups (class 2) and amino groups (class 3). The following quantities were chosen identical for all proteins: pK_2 = 7,pK_3 = 10,n_2 = 20 and n_3 = 60.
The number of groups and the pK of class 1 were varied.
Curve A: pK_1 = 4,0 n_1 = 100; Curve B: pK_1 = 4,7, n_1 = 40;
Curve C: pK_1 = 4,7 n_1 = 72; Curve D: pK_1 = 4,7, n_1 = 100.

With n_1 = 73 and n_2 = 19, read from the peak positions, curves are calculated with various values of Δ pK. It is seen that the shape of the curve is very sensitive to variation of Δ pK, while only a relatively small change in peak position is found. The curve with Δ pK = 2.3 has the same shape as curve C; the peak however being one group displaced to the right. Thus n_1 and n_2 are not 73 and 19, but 72 and 20, respectively. With these new values of n_1 and n_2 and Δ pK = 2.3 the curve is recalculated and in this case a curve is found which is of course identical with curve C. When this procedure is used for a real experimental curve it can be repeated, if necessary.If w has a constant value the calculated

curve will be shifted vertically because of the term 0.868w (and a possible constant for other bound ions) in equation (2.3.5); the shape and peak position, however, will be the same. From the vertical shift the apparent w (sum of real w and the constant due to other bound ions) can be obtained. When the distance between calculated and experimental curve is not constant there are deviations from our symplifying premises. Such a variable distance may indicate that some conformational change takes place or that the assumption of identical groups is not quite justified, or that the number of other bound ions is not a linear function of the charge, etc.

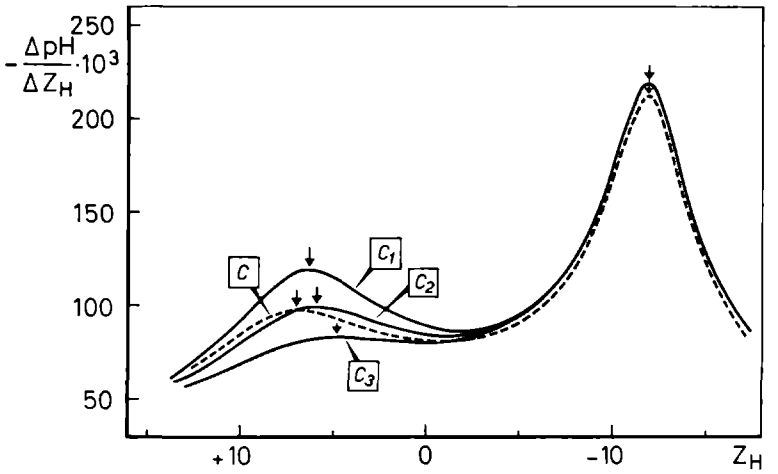

Fig.2. Determination of the number of carboxyl groups and imidazole groups by means of successive approximation.
Curve C is identical with that of Fig. 1, and is considered as experimental curve. Curves C_1, C_2 and C_3 were calculated with $n_1 = 73$, $n_2 = 19$, $n_3 = 60$, $pK_2 = 7$ and $pK_3 = 10$ but with $pK_1 = 4.5$, 4.7 and 4.9 respectively.

2.4 Methods

pH measurements were carried out with a Radiometer equipment consisting of a pH meter type PHM 26 C, equipped with a scale expander, and a glass electrode, type G 202 B combined with a calomel electrode, type K 401, or an Ag-AgCl electrode. These electrodes were preferred to the Radiometer micro-electrodes because of their much smaller response time. The system was calibrated using standard buffers (National Bureau of Standards). The buffer readings after titration were always about 0.01 pH higher than before. This systematic error is probably caused by some disturbance of the liquid junction by the protein molecules, since the original reading was re-obtained after several hours in buffer. In practice, this small effect had no influence on the differential curve. In the region below pH = 4 the calomel electrode is still less reliable. Here the Ag-AgCl reference electrode was used.In this case no systematic error was found and the reproducibility of buffer readings before and after titration was about 0.002 pH. The titration vessel, thermostated at $25^{o} \pm 0.02^{o}$, usually contained 3 ml of the solution. The titrations were carried out under nitrogen and the solutions were vigorously stirred by a magnetic stirrer. The titrant was HCl or carbonate-free NaOH, both having the same concentration of about 0.05 M. Constant ionic strength during titration was achieved by adjusting the protein solution with KCl to the ionic strength of the titrant. The amount of KCl diffusing into the solution from the calomel electrode during a titration was negligible, even at low ionic strength. A schematic representation of the automatic equipment is given in Fig. 3. It consists of the following units: a motordriven microburette (Metrohm, type E 475); the above mentioned equipment for measuring the pH; a high resolution digital voltmeter (Solartron type LM 1440-3); a convertor (N.V. Peekel, Rotterdam) to make the readings of the digital voltmeter suitable for printing; a printer (Addo-X); a potentiometer (p) by which the voltmeter reading is adjusted to 100.00 mV per pH unit; a motordriven programmer and a motordriven timer. The three motors used are synchronous motors (Halstrup type M 64/10) with adjustable gear box allowing revolution speeds of 0.01 to 10 r.p.m. The accuracy of the plunger-type burette with a maximum content of 0.5 ml is 0.1 $^{o}/_{o}$ per 0.01 ml titrant added. It was equipped with a very fine capillary dipping into the solution. One revolution of the burette-drive corre-

sponds with 0.01 ml of added titrant. When usual protein concentrations are employed (10 to 20 mg per ml), the corresponding change in proton charge ΔZ_H is 0.5 to 1.0 for proteins with a mol. weight of ca 65.000.The controlling unit of the whole equipment is the programmer. It consists of three coaxial disks. On each disk studs have been mounted which control, by means of micro-switches, the several units. One disk with one stud commands the timer which in turn commands the burette. The second disk equipped with ten studs orders the digital voltmeter to measure the pH ten times with equal intervals of a few seconds. At the same time these readings are printed. The third disk gives order to the printer to add the pH-readings giving directly the mean value. In this way pH differences can be measured with an accuracy of a few ten-thousandths of a pH unit. At the bottom of Fig.3 one titration cycle is shown. All commands indicated are given by the programmer, except the burette stop command which is given by the timer.

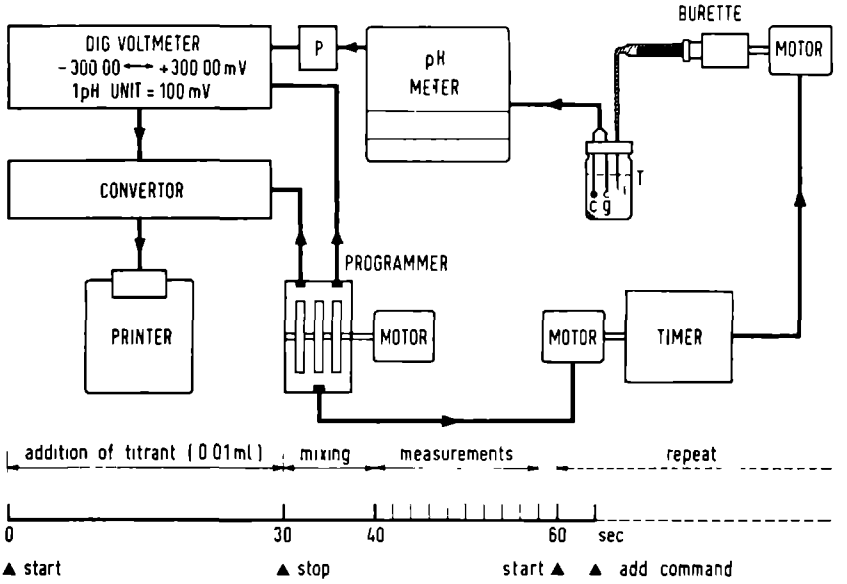

Fig.3. Schematic diagram of the automatic titration equipment.
c : calomel electrode
g : glass electrode
T : titration vessel
For other details see text.

It will be illustrative to compare the accuracies obtained in measuring Δ pH and ΔZ_H with those required. For most proteins with a mol. weight of about 65.000 $\Delta pH/\Delta Z_H$ is ca. 100.10^{-3} near pH = 7. Suppose we wish to measure this quantity within $1^o/o$. Then both Δ pH and ΔZ_H must be measured within about $0.5^o/o$, in other words Δ pH must be measured, when $\Delta Z_H = 1$, within an accuracy of 5×10^{-4}. So it is seen that the requirements for a good measurement are fulfilled.
Thermodynamic equilibrium was assumed when forward and backward titrations were identical within the limits of experimental accuracy. From a large number of titrations the accuracy of the position of a peak in the differential curve (inflection point in the normal curve) appeared to be $\pm$ 0.2 groups. All curves are the mean of at least one forward and one backward titration curve. Calculation of the differential titration curves were executed on the computer IBM system 360/50 using a Fortran IV program.

2.5 Materials

Crystalline bovine serum albumin (BSA) was obtained from the Nutritional Biochemicals Corporation. The protein solutions were deionized by dialysis or by passing them over a mixed bed ion-exchange column (amberlite IRA 400 and IR 120) using a recycling system - see chapter 4 -. The recycling system consists of a peristaltic pump and a small column with a content of about 4 ml. This system has the great advantage that protein solutions can be deionized completely with negligible dilution. The time needed for deionisation was measured by conductivity measurements - see chapter 4 -. It is essential that, before deionizing by means of the column, the protein solutions are made nearly saltfree by dialysis because the rate of exchange of anions by hydroxyl ions is greater than that of cations by hydrogen ions. So, if the solution contains too much salt, locally strong basic regions will be formed. Consequently denaturation will then take place. At high salt concentration this effect manifests itself in the high pH (to pH = 12) of the effluent directly after the system has been started.
Concentrations were determined by drying at 105^o in air to constant weight. For the titrations freshly prepared solutions of a concentration of one weight percent were used which were brought to the desired ionic strength with KCl.

2.6 Results and discussion

Fig. 4, curve A, gives the results for BSA in the neutral region; these are based on a molecular weight of 69,000. The distance between the peaks is 16.3 ± 0.3 groups. The protein has one α-amino group with a pK of about 8.0. It can be calculated that about one half of this group has not yet been titrated at the second peak. Calculations show - see further - that the left peak is shifted about 0.6 group to the right. So we find 17.4 ± 0.3 groups in the neutral region, 16.4 ± 0.3 of which are imidazole groups. Tanford et al.[10] found 17 imidazole groups, when their results are converted to a molecular weight of 69,000 (a number of 18 is mentioned in a latter publication[2]). From the amino acid analysis of Stein and Moore[11] and of Spahr and Edsall[12] - see table 1 - 18 histidine residues can be calculated. So it seems possible that one or two imidazole groups are not able to exchange protons with the solvent in

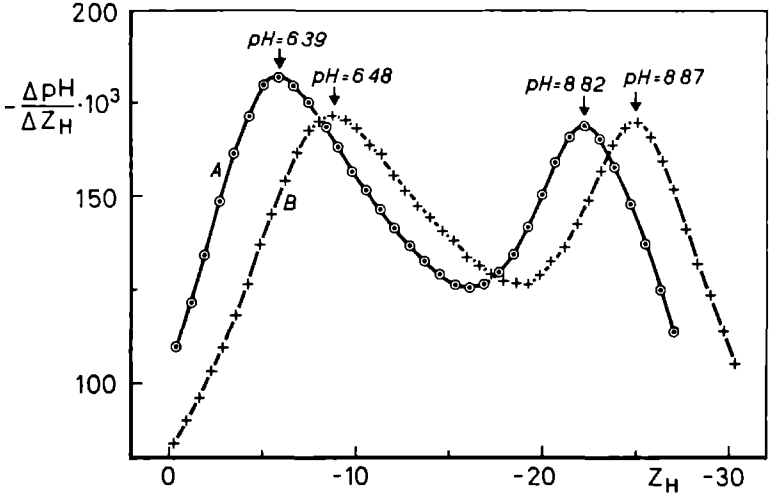

Fig.4. Differential titration curves of BSA, based on a mol. wt of 69,000.
Curve A : Deionised BSA, concn. 1°/o, I = 0.062. (KCl)
Curve B : Dialysed BSA, concn. 1°/o, I = 0.062 (KCl)

Table I

Titratable groups of BSA ; M 69,000.

Z_m titration value	Z_m amino acid analysis	n_c titration value	n_c amino acid analysis	n_{His} titration value	n_{His} amino acid analysis	Reference number
103.5		106.6				15
102		106.1		18		2,10
99		104.5		16.5		This thesis
	103		91		18	11
	107		105		18	12

the neutral region. This idea is supported by the experiments of Foster et al.[13,14] who found that the isomeration of BSA at low pH is accompanied by an uptake of protons. Therefore some buried imidazole groups might be involved in this process.

Fig.5 gives the normal pH vs. Z_H titration curve in the range below the isoionic pH. The curve levels off at pH = 2 and reaches a maximum value of Z_m = 99. This constant level is usually not reached when a calomel electrode is used. In that case one has to extrapolate to low pH values to obtain Z_m - see, for instance, the titration curve of BSA given by Sogami and Foster[15] -. We have succeeded in reaching Z_m, using an Ag-AgCl electrode as reference electrode. The observed value of Z_m, including the possibly masked groups becoming titratable at acid denaturation, is somewhat smaller than the titration values reported by others - see table 1 -. It is also somewhat lower than the value based on the amino acid analysis of Stein and Moore[11] and even remarkably different from the data of Spahr and Edsall[12]. The latter value,

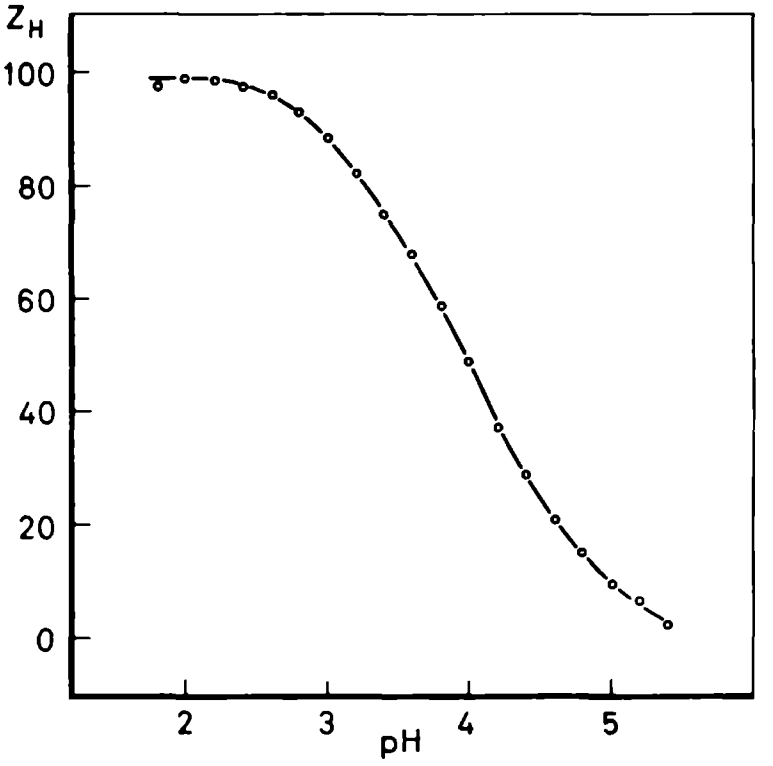

Fig.5. The normal titration curve of deionized BSA at 25° , for the range below the isoionic pH.
I = 0.505 (KCl), concn. 2°/o.

however, exceeds also both other reported titration values of Z_m. The difference with the titration values of others might be explained by the fact that they are obtained by extrapolation, while the value reported by us is obtained from measurements. The difference between Z_m = 99 and the two reported values based on the amino acid analysis is rather high. Especially the disagreement with the value reported by Spahr and Edsall is rather striking. It must be noticed, however, that these authors also find that the number of positive groups exceeds the number of carboxyl groups n_c. This does not seem very likely because in that case the left peak in the differential titration curve - Fig.4 - must be found at positive Z_H values. This peak, however, is found at Z_H = - 6 ± 0.2. In

addition the sum of the arginine and lysine residues - equal to Z_m minus the 18 histidines and the α-amino group - found by us is 80. This value is in better agreement with that reported by Stein and Moore[11](84) than with that of Spahr and Edsall[12](88).

From the left peak position (applying $n_c = Z_{max} - Z_p$) a number of 105 ± 0.5 carboxyl groups is approximately found. In order to find out if there might be a shift to the right, we calculated the curve in the region of this peak with Z_m = 99 and with several values for the number of carboxyl groups (n_c), of imidazole groups (n_I) and of Δ pK = $pK_I - pK_c$. Contributions of other groups were were neglected. As can be seen in Fig.6, only with Δ pK = 2.8 a curve was obtained having about the same shape as the experimental curve, while subsequently the positions of experimental and calculated curve became identical with n_c = 104.4 and n_I = 16.4.

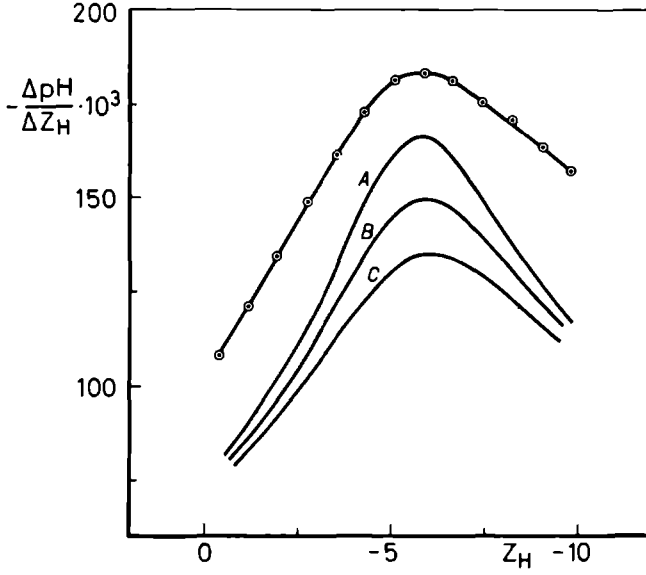

Fig.6. Calculated differential titration curves of BSA compared with the experimental curve.
Upper curve: Part of curve A of Fig. 4.
Curves A, B and C were calculated, assuming 104.4 carboxyl groups and 16.4 imidazole groups, but with Δ pK ($= pK_I - pK_c$) = 2.9, 2.8 and 2.7 respectively.

(The calculated curves for other values of n_I are omitted in Fig.6 to avoid overcrowding of the figure). The values of $\Delta pK = 2.8$ is in good agreement with the pK values given by Tanford[10], viz. $pK_c = 4.0$ (anomalously low) and $pK_I = 6.9$. The calculation clearly shows that the first peak is about 0.6 group shifted to the right, and thus that n_c is 104.4 ± 0.5 instead of 105 ± 0.5. The discrepancies between this value of n_c and the values reported by Tanford[10], Sogami and Foster[15] are probably within the experimental error. The agreement with n_c predicted by the amino acid analysis of Spahr and Edsall[12] is very good. The value given by Stein and Moore[11] is probably too low because their estimation of the amide content is very likely too high. It should be noted further that, if one or two imidazole groups are masked and if they are liberated at low pH, the number of carboxyl groups is not 104.4 ± 0.5 but also one or two less.

From Fig.6 the value of the constant 0.868w can also be found. It is the distance between the experimental and calculated curve B, being $(33 \pm 5) \times 10^{-3}$. Calculation of 0.868w, neglecting chloride-binding and using $R = 30$ Å and $a = 32.5$ Å, yields 33×10^{-3}. Since chloride-binding causes a decrease of w of about 20°/o[2,10], this agrees reasonably well with the experimental value.

The position of the minimum of curve A in Fig.4 is rather striking. If all the imidazole groups have the same pK, and if w is constant in the pH region in which these groups are titrated, then the minimum will be expected about midway between the two peaks. Because Tanford and Buzzell[16] found constancy of w between pH = 4 and 10.5, the excentrical position of the minimum can only be caused either by non-identical imidazole groups with different pK, or by some conformational free energy change accompanying the dissociation of the imidazole groups and superimposed on the free energy change of the dissociation process. The former possibility is followed by Decker and Foster[17]. They accept 10 imidazole groups with pK = 6.50 and 7 with pK = 7.50. The latter possibility is supported in our opinion, by the results of Leonard et al.[18]; they found - from optical rotation measurements - that some conformation change takes place between pH = 7 and pH = 9, thus roughly in the same range in which the imidazole groups are titrated. The change in optical rotation at $\lambda = 313$ mμ found by them was about 5°/o. These results have been confirmed by us. The temperature dependence of the differential titration curve - see further - also

supports this idea. Moreover Klotz et al.[19] have found that in this region the number of binding sites for anionic and neutral dyes increases strongly which is also an indication of anomalous behaviour of the protein in that pH region. The same increasing number of sites for Ca^{++} ions has been found by Harmsen[20]. A similar anomalous behaviour is found by Benson and Hallaway[21] studying the exchange of hydrogen with deuterium. This investigation revealed that between pH 3 and 8.5 the number nonexchangeable hydrogens is about 150 and outside this region zero. A quantitive explanation of this phenomenon is not possible so far because of the lack of knowledge about this conformation change.

Curve B of Fig.4 shows the results for BSA which had been made isoionic by dialysis against 0.0624 m KCl. The whole curve is about three groups displaced to the right, with respect to curve A. It is known that BSA contains a few moles of fatty acid per mole[9,15]. It is also known that they cannot be removed by dialysis. From the shift of about three groups we conclude, for our sample, that there are three molecules fatty acid per BSA molecule which are removed by a mixed bed ion-exchange column. The presence of the three extra carboxyl groups also corresponds quantitatively to the displacement of the isoionic point. The number of carboxyl groups titrated at the isoionic point is for both proteins about 99 ($= Z_m$) and so $\alpha_c = 99/104.4$ for deionised albumin and $\alpha_c = 99/107.4$ for dialysed albumin. When these values are substituted in equation (2.2.2), two equations are obtained from which we calculated Δ pH = 0.19, while experimentally 0.18 was found. In this calculation the reasonable assumptions are made that the extra carboxyl groups have about the same pK as the other carboxyl groups and that both proteins have the same total charge Z at the isoionic point; in other words that the number of bound chloride ions is the same for both proteins. The difference in height of the left peak is a third indication for the presence of extra carboxyl groups in dialysed albumin because, as can be seen from eqn. (2.3.5), an increase of n_c results in a lower value of dpH/dZ_H.

In Fig.7 finally the temperature dependence is shown of the differential titration curves of column defatted BSA. It is seen that in going to lower temperature the left peak increases strongly and is shifted to more positive Z_H-values. This behaviour is in accord with the expectations because going to lower temperature $\Delta pK = pK_I - pK_c$ becomes larger

(the enthalpy differences ΔH_I and ΔH_c are about 1 and 7 kcal/mole resp.), which results in an increased peak height and a smaller shift to the right (cf. Figs. 1 and 2). Calculations show that Δ pK increases with 0.20 (0.14) in going from 30^o to 15^o (15^o to 5^o). From this Δ pK changes a mean difference of 5.3 kcal/mole in ionisation enthalpy is calculated between the imidazole and carboxyl group which is in good agreement with the results of Tanford et al.[10] viz. ΔH_I = 7 kcal/mole and ΔH_c = 1 kcal/mole.

The right peak also increases; the increase is, however, less than that of the left peak likely because the pK of the α-amino group is changed in about the same way as the pK of the imidazole groups.

Another remarkable effect is the shift of the minimum. We have no detailed explanation for this phenomenon, but we have the feeling that

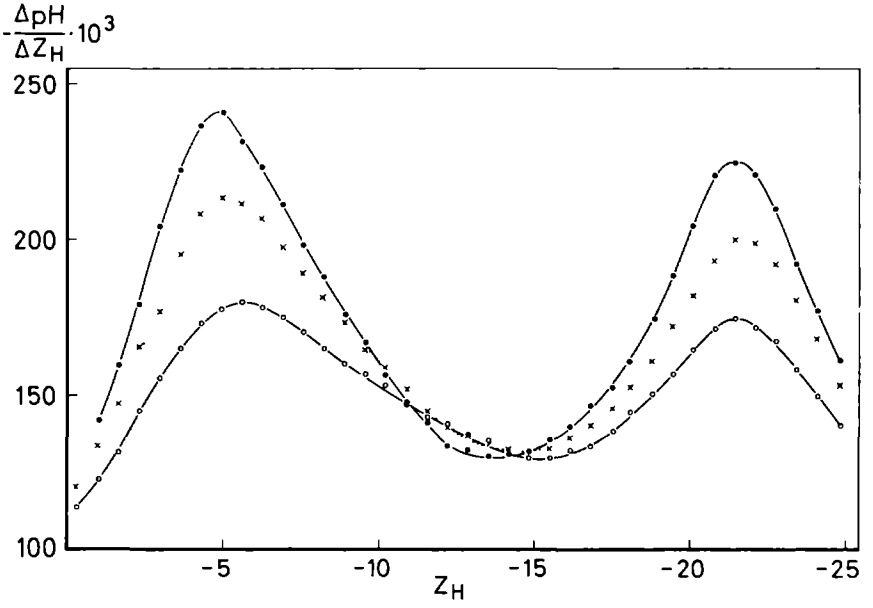

Fig.7. Differential titration curves of BSA at 30^o (o), 15^o (x) and 5^o (•). Concn. 2°/o, I = 0.06 (KCl).

it is related to the above mentioned conformation change which takes place in this Z_H-region. This shift is, in our opinion, also an argument against the existence of two different classes of imidazole groups[17] because, if so, it seems likely that the pK values of both classes will vary in the same way with temperature and consequently no shift would be observed.

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Chapter 3

STUDY OF THE BOHR GROUPS AND OF THE NUMBER OF MASKED IMIDAZOLE GROUPS OF BOVINE HEMOGLOBIN.

3.1 INTRODUCTION

The normal or alkaline Bohr effect of hemoglobin is the phenomenon that protons are liberated when oxygen is bound, and so that the oxygen affinity increases with increasing pH. This effect is found between about pH 6 and 9. There is also a reversed or acid Bohr effect which is found roughly below pH 6. Reviews on these phenomena of linked functions have been given by Wyman[1,2]. The total Bohr effect can be described by assuming that two groups per chain change their pK upon oxygenation; one with a pK-change from about 8 to 7 is responsible for the normal Bohr effect, the other with a pK going from about 5.5 to 6 for the reversed Bohr effect. It has been supposed that these groups are located near the heme group because the influence of oxygen binding on the ionisation of these groups then becomes understandable. However, since myoglobin[3] and also the isolated α and β chains[4,5] do not show an appreciable Bohr effect this explanation seems improbable. It is more likely that the conformational change accompanying oxygenation of Hb[6,7,8] causes the change in pK of the two Bohr groups.

The identification of these protonic groups is still a matter of discussion. From an analysis of the temperature dependence of the proton binding of Hb and HbO_2 Antonini et al.[9] concluded that the alkaline Bohr group probably is an imidazole group and the acid Bohr group probably a carboxyl group. Tanford and Nozaki, however, in view of their titration results, do not exclude the possibility that the alkaline Bohr group is an α-amino group. Hill and Davis[11] have given strong evidence that the α-amino group of the α-chain of human carbon monoxide hemoglobin (HbCO) has a pK near 7 which is one unit lower than the expected value, and they also suggested this group might be the alkaline Bohr group.

This chapter presents a study of the Bohr effect of bovine hemoglobin before and after reaction with 1-fluoro-2,4-dinitrobenzene

(FDNB) by means of normal, differential and difference* hydrogen ion titration curves; it is an attempt to identify the alkaline Bohr groups.

3.2 MATERIALS AND METHODS

Deoxygenated bovine hemoglobin was prepared by the method of Drabkin[12] modified in the way as performed by Chipperfield, Rossi-Bernardi and Roughton[13]. The concentration of the hemoglobin solutions obtained was about 70 mg per gram solution. The solution was diluted with deionized water to a concentration of about 2.5°/o. After exhaustive dialysis the solution was subsequently deionized by passing it over a mixed bed of ion-exchange column - see chapter 2 -.The concentration of the stock solution so obtained was about 2°/o; it was stored at 4° under purified argon. Oxygenated hemoglobin was obtained by passing purified oxygen over the sample in the titration vessel. Concentrations were determined by weighing after drying at 105°C to constant weight. DNP- (2,4 -dinitrophenyl) substituted oxyhemoglobin (DNP-HbO_2) was prepared by the procedure decribed by Hill and Davis[11]; the solutions were dialysed and deionized as described above; the concentrations of the stock solutions were determined by the dry weight method. Deoxygenated DNP-substituted hemoglobin (DNP-Hb) was prepared by passing purified argon over a sample of the stock solution in the titration vessel. FDNB was obtained from the Nutritional Biochemicals Corporation. The titration procedure was basically the same as described in chapter 2.

All curves are the mean of at least one forward and one back titration curve. The most critical are the $\Delta pH/\Delta Z_H$ vs. Z_H curves. The

* Note:
Often the term differential titration curve is used to indicate the curve which represents the difference in protons bound by Hb and HbO_2. In our definitions - see chapter 2 - this terms stands for the curve which gives the slope of the normal titration curve vs. Z_H, the mean proton charge.
We will call the curve giving the difference in protons bound the difference titration curve.

overall accuracy of Δ pH/ ΔZ_H was for one sample better than 1°/o and for the mean of several different samples better than 3°/o.
The desired ionic strength was adjusted with KCl. Solid phosphate -free KCl (Merck, suprapur), instead of a KCl-solution, was added to avoid contamination with CO_2. The mol. wt. of HbA (Dutch cattle is homozygote for HbA) was taken equal to 65.000, based on the amino acid sequences given by Schroeder et al.[14,15] for the α and β chain of bovine HbA. The same value is given by Antonini[16]. Titrations of Hb and HbO_2 were carried out with the same sample in the titration vessel. The same was done for DNP-Hb and DNP-HbO_2. In this way non-systematical concentration errors are avoided making comparison of the curves for oxygenated and reduced hemoglobin more reliable. Before starting the titration of Hb(DNP-Hb) or HbO_2(DNP-HbO_2) purified argon or oxygen was passed over the solution to constant pH. All titrations were performed at 25 ± 0.02°. Calculation of the titration curves were executed on the computer IBM system 360/50, using a Fortran IV program.

3.3 RESULTS AND DISCUSSION

As has been pointed out in the preceding chapter the analysis of the differential titration curves is based on the following set of equations

$$pH = pK_i + \log \frac{\alpha_i}{1-\alpha} - 0.868wZ \qquad (3.3.1)$$

$$Z_H = Z_m - \sum_i n_i \alpha_i \qquad (3.3.2)$$

and

$$-\frac{dpH}{dZ_H} = \frac{1}{2.303 \cdot \sum_i n_i \alpha_i (1-\alpha_i)} + 0.868w =$$

$$= \frac{1}{2.303.S} + 0.868w \qquad (3.3.3)$$

A calculated differential curve ($-dpH/dZ_H$ vs. Z_H) can be obtained from these equations when a proper choice is made for the pK_i of the various classes of groups, their numbers of groups n_i and for Z_m. If w is a constant, a comparison of this curve with the experimental one then gives information about the pK_i's and the value of w.

In the calculations the numbers of the groups titrated in the basic region were taken from the amino acid analysis[14,15]. Z_m was taken equal to the sum of the groups titrated in the neutral region and the number of groups from the basic region which become positively charged upon proton binding. The number of carboxyl groups n_c was calculated according to the relation $n_c = Z_m - Z_p$ in which Z_p is the Z_H value at the position of the left peak in the differential titration curve. As has been shown in chapter 2 Z_p can be considered as the "titration endpoint" of the carboxyl groups within a fraction of one group provided the peak is sufficiently pronounced. This is the case for deoxygenated hemoglobin A, as can be seen in fig.1, which shows the results for Hb and HbO_2.Before giving details about these calculations, we will first discuss some qualitative conclusions that can be drawn from fig.1. The presence of the normal Bohr groups is directly seen from the difference in height of the two right peaks at $Z_H = -10.3$. Clearly the contribution of these groups to S eqn. (3.3.3) decreases on oxygenation resulting in an increased peak height for HbO_2. This contribution to S is shifted to higher Z_H-values and is found as a lowering of the minimum at about $Z_H = +1$. So, there must be some groups in this pH region which change their pK to a lower value as a result of oxygenation. The presence of the acid Bohr groups is found in the lowering of the left peak and its shift to $Z_H = +12.3$ on oxygenation. These groups must give an enlarged contribution to S for HbO_2 at $Z_H = Z_p$. This means that the α of these groups in HbO_2 must be nearer to $\alpha = 0.5$ than in Hb because $n\alpha(1-\alpha)$ reaches its maximum for $\alpha = 0.5$. From the shift of the peak upon oxygenation it follows that the α of the acid Bohr groups in HbO_2 is smaller than in Hb at Z_p giving a more positive Z_p value. So these groups must have a higher pK in HbO_2 than in Hb. According to Antonini et al.[9] we assume that they are carboxyl groups. A more quantitive approach can be obtained by estimating the numbers of groups in each class in the following way. Since the distance between the two peaks in Hb is 21.6, a number of 22 titratable groups in the neutral region was chosen. If four of these groups are α-amino groups,

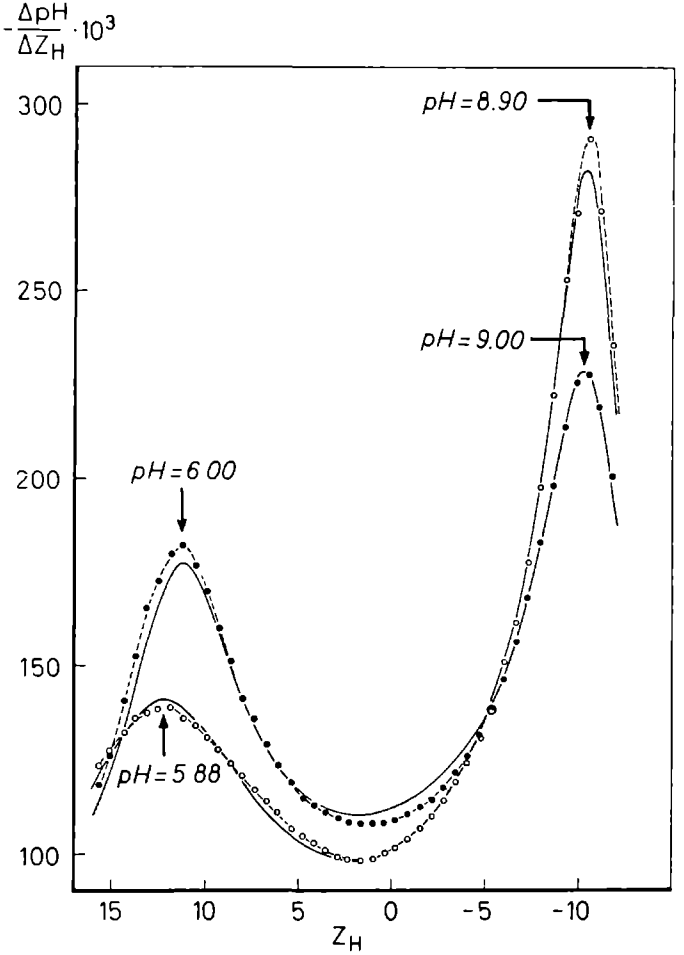

Fig.1. Differential titration curves of oxygenated (o) and deoxygenated (●) hemoglobin at 25°. Protein concn. 20 mg/ml; I = 0.13 (KCl). Drawn lines are calculated, using the parameters of table I.

18 titratable histidines remain; Z_m for the native protein will then be 84 (viz. 48 Lys, 18 His, 14 Arg and 4 α-amino groups). The number of titratable carboxyl groups, the acid Bohr groups included, follows from $n_c = Z_m - Z_p = 84 - 11.3 = 72.7$. There are 54 Asp, 34 Glu, 4 terminal carboxyl groups and 8 heme carboxyl groups. So 27.4 Asp and Glu residues are present as amides. The amino acid analysis gives a number of 24[14,15]. Possibly the number of amide groups found from the amino acid analysis is wrong since for human hemoglobin, which has been studied more extensively, the agreement is perfect[17].With these parameters we have made an attempt to calculate the whole titration curve between about pH 5 and 9. The basic groups (48 Lys, 10 Tyr, and 2 Cys) were assumed to be all titratable and were taken as one class. Although actually the pK values of these 3 classes are probably not equal and moreover some of the Tyr residues are likely not able to exchange protons, the error will be small because the Lys class is by far the largest one; moreover the same assumption is made both for HbO_2 and Hb. In addition Z_m and so the position of the peaks is not altered by a varying number of titratable Tyr residues. The pK values and numbers of groups that had to be used to obtain a reasonable good fit are given in Table I.

These parameters could be combined with a constant value of 0.015 for 0.868w, in Hb as well as in HbO_2. It is reasonable to assume that w will be constant since, as has been found from sedimentation measurements by Chiancone and Gilbert[18], the protein does not dissociate in the pH region of our titrations. The low value of w, however, clearly indicates that the commonly used formula - eqn.(1.4.5) - for the electrostatic interaction factor w, which yields $0.868w = 0.029$, is not valid in this case. A similar low value, as found by us, was reported by Bucci, Fronticelli and Ragatz[19] for the ionisation of the heme H_2O in human MetHb. In Fig.1 the calculated differential curves are given as drawn lines.

Fig.2 shows the experimental and calculated normal titration curves. Comparison shows that the differential titration is much more sensitive for discrepancies between calculated and experimental curve. From table I it is seen that in Hb eight groups of pK = 8 are necessary to obtain a good fit between the experimental and calculated curves. In HbO_2 four of these eight groups, the alkaline Bohr groups, have changed their pK to 7, while the pK of the other groups remains unchanged. An attempt to

Table I

Numbers of groups and their pK-values used to obtain calculated, normal, differential and difference titration curves.

numbers of groups in unsubstituted hemoglobin	numbers of groups in DNP-substituted hemoglobin	pK_I-values before oxygenation	pK_I-values after oxygenation
68.6	68.6	4.25	4.25
4	4	*5.30*	*5.80*
14	*13*	7.10	7.10
4	*2*	*8.00*	*7.00*
4	4	8.00	8.00
60	60	10.65	10.65

identify the alkaline Bohr groups is described below. It should be noted that the pK values in table I were found by trial and error and are somewhat arbitrary. A still better fit by small changes of the pK's remains of course possible. The number of 18 titratable imidazoles means that 14 of the 32 histidine residues are masked. Nozaki - see ref.20 - has found 16 buried groups in bovine methemoglobin which is in reasonable agreement with our value. Tanford and Nozaki[10] reported a value of about 16 unavailable groups in human hemoglobin and about 12 in horse hemoglobin*. Bucci, Fronticelli and Ragatz[19] reported a value of 14 to 18 buried histidine residues in human hemoglobin. Beychok and Steinhardt[21], Steinhardt et al[22], and Geddes and Steinhardt[23] found that, when human and horse methemoglobin or

* Note:
The same value for horse hemoglobin was found by us.

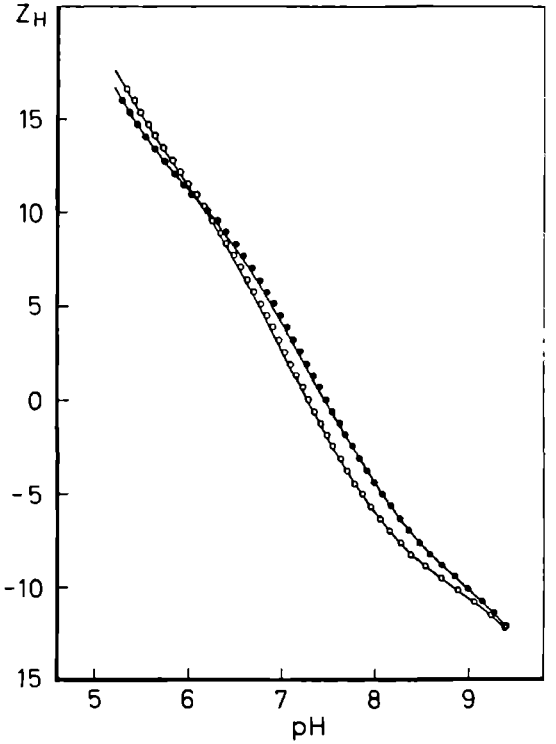

Fig.2. Normal titration curves of oxygenated (o) and deoxygenated (•) hemoglobin, based on the same experimental data as the curves of Fig.1. Lines are calculated, using the parameters of table I.

carbon monoxyde hemoglobin are exposed to acid at about pH = 3, the number of unmasked groups exceeds largely the above mentioned values. They suppose that besides imidazole groups other more basic groups are masked too, for instance ε-amino groups or ionized phenoxy groups. We have, however, some objections to this supposition. For, if there are uncharged ε-amino groups, Z_m will be smaller and applying the relation $n_c = Z_m - Z_p$ a too low value for n_c will be obtained. The

same is true if there are ionized phenoxy groups, because in that case n_c will be equal to $n_c = Z_m - n_{phenoxy} - Z_p$ which results also in a too low value for n_c. For human hemoglobin now the relation $n_c = Z_m - Z_p$ is fully applicable with the normal value for Z_m. It seems more likely to us that the extra masked groups are ionized carboxyl groups, for only then the above relation will not be affected.

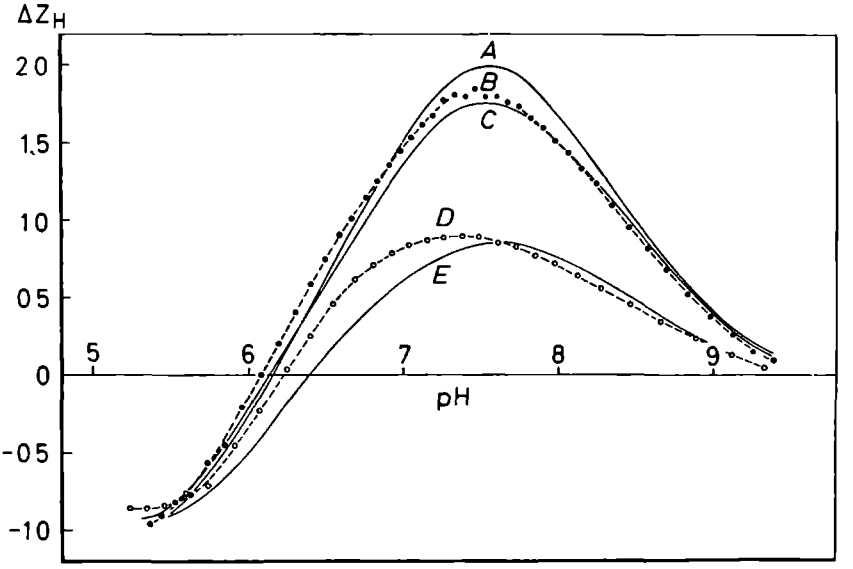

Fig.3. Difference titration curves (ΔZ_H vs pH) of hemoglobin (curve B) and of DNP-substituted hemoglobin (curve D).
Curves C and E were calculated according to Eqn. (3.3.4), curve A according to the commonly used simplified Eqn.(3.3.5), using the parameters of table I.

Fig.3 shows the observed and calculated difference titration curves. Curve C is calculated according to

$$\Delta Z_H = (\Sigma_i n_i \alpha_i)_{Hb} - (\Sigma_i n_i \alpha_i)_{HbO_2} \qquad (3.3.4)$$

The agreement with the experimental curve B is quite satisfactory especially above pH = 7.6, where the acid Bohr groups do not contribute to ΔZ_H. Curve A is calculated according to the equation which in commonly used[24] (3.3.5)

$$\Delta Z_H = 4(\alpha_{N.B} + \alpha_{A.B})_{Hb} - 4(\alpha_{N.B.} + \alpha_{A.B})_{HbO_2}$$

in which $\alpha_{N.B.}$ is the α of the normal Bohr groups and $\alpha_{A.B.}$ that of the acid Bohr groups. Eqn. 5 can be obtained from Eqn. 4 by assuming that only the ionisation of the Bohr groups is affected by oxygenation. This is an approximation because at constant pH the charge of Hb is ΔZ_H more than that of HbO_2, which implies that pK_{app} (= pK_{int} - $0.868wZ_H$) of all groups will differ $0.868w\Delta Z_H$ in the two states. Consequently the α of all groups in Hb and HbO_2 is different. As is shown by the two calculated curves this difference in charge counteracts the normal Bohr effect.

It has been suggested by Hill and Davis[11] and by Rossi-Bernardi and Roughton[24] that the Linderstrøm-Lang approach, as has been used here, is too simple, at least for the alkaline Bohr groups. These authors find w nearly zero in human and horse hemoglobin. If this would be true for all groups, it would mean that the neutralizing effect of ΔZ_H on the normal Bohr effect is zero too. However, for bovine hemoglobin we could not obtain reasonable calculated curves with w = 0. So, even if w would be zero for the Bohr groups, it seems very likely that this is not so for all other groups. Therefore the counteracting effect remains although it is small thanks to the low value of w.

In Fig.4 (curve A) the observed $(\Delta pH)_{Z_H}$ (= pH_{Hb} - pH_{HbO_2} at the same Z_H) is plotted vs. Z_H, while curve B represents an attempt to calculate this curve with the parameters of table I according to:

$$(\Delta pH)_{Z_H} = \left(\Delta \log \frac{\alpha_{Im}}{1 - \alpha_{Im}}\right)_{Z_H} \qquad (3.3.6)$$

These curves are similar to those found for human and horse hemoglobin[9]. The position and height of the maximum is about the same. The only difference is that they are less symmetrical.

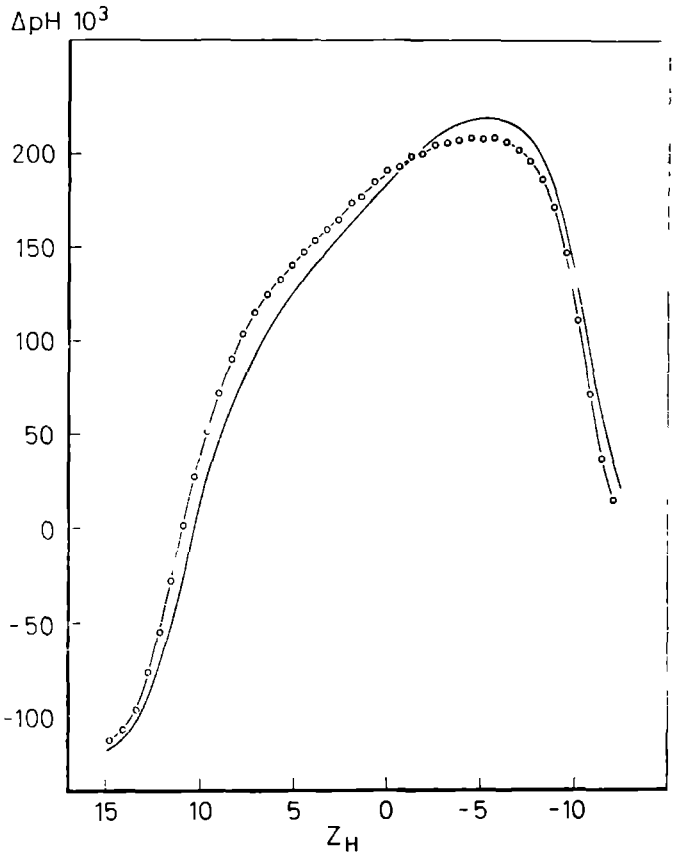

Fig.4. Difference titration curves (ΔpH vs $\bar{Z}_H$) of unsubstituted hemoglobin (o). Drawn line is calculated using the parameters of table I.

As has been remarked in the introduction, the identity of the alkaline Bohr groups is still a matter of discussion. It was also mentioned that Hill and Davis[11] have shown that the α-amino groups of the α-chains have a pK near 7 in human HbCO. This pK value was obtained by measuring the pH-dependent rate of reaction of FDNB with these α-amino groups. However, it had not yet been proved that these groups have a pK near 8 in reduced hemoglobin, which is required when these groups are the Bohr groups. Neer and Koningsberg[25] have found that

FDNB reacts much faster with the α-amino group of the α-chain than with that of the β-chain, and that DNP-substituted hemoglobin has lost cooperative interactions. Therefore we have made an attempt to elucidate the problems around the identification of the alkaline Bohr groups by studying the differential titration curves of DNP-Hb and DNP-HbO_2.

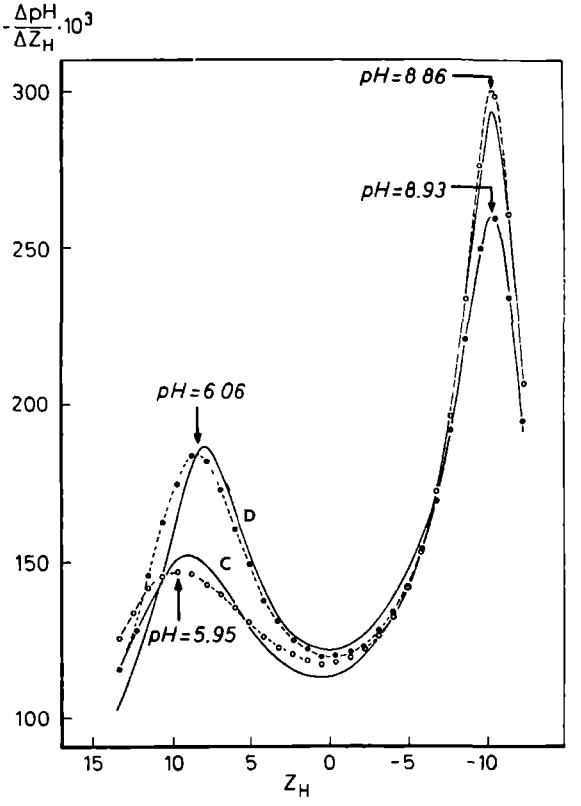

Fig.5. Differential titration curves of oxygenated (o) and deoxygenated (•) DNP-substituted hemoglobin at 25°.
Concn. ratio (moles) FDNB : HbO_2 = 4 : 1.
Reaction time 1 h at 20°, pH = 7.2. Protein concn. 20 mg/ml; I = 0.13 (KCl). Drawn lines are calculated using the parameters of table I.

Fig.5 shows the results of DNP-HbO_2 and DNP-Hb. The substituted product was obtained by reaction between FDNB and HbO_2 during one hour in a concentration ratio of 4 FDNB to 1 hemoglobin at 25^o and pH = 7.2. The FDNB which had not reacted was removed by exhaustive dialysis at 4^oC. The two peaks in the curve for DNP-Hb are situated at $Z_H = +8.4$ and $Z_H = -10.3$. The distance between the two peaks in DNP-Hb is 18.7; consequently about three groups have been blocked. These must be groups in the neutral region because, if residues as lysine would have reacted, the two right peaks would not have the same position (at $Z_H = -10.3$) as in Hb and HbO_2.
Comparing the curves for Hb and HbO_2 with the curves for DNP-Hb and DNP-HbO_2, it is seen that the right peak in the curve for DNP-Hb is increased with respect to the corresponding peak for Hb, while the right peak in the curve for DNP-HbO_2 is only slightly different from the one for HbO_2. This can be explained by assuming that some of the alkaline Bohr groups have been blocked. Then, on substitution, the contribution of these groups to S - eqn. (3.3.3) - in Hb at $Z_H = -10.3$ is decreased which results in an increased peak height in DNP-Hb. The contribution to S of these groups in HbO_2 at $Z_H = -10.3$ is because of the pK-change much smaller, so that the peak height in DNP-HbO_2 is hardly changed. The decreased difference between the two minima near $Z_H = 0$ is in accord with this explanation because reversely in HbO_2 the normal Bohr groups contribute here much more to S than in Hb. The increase of the two minima also corresponds with the disappearance of these groups.

The height of the two left peaks for Hb and HbO_2 is hardly affected by substitution. The little increased height of the left peak for DNP-HbO_2 is likely caused by the disappearance of the blocked normal Bohr groups which have some contribution to S in HbO_2 at the peak position. So it may be concluded that the pK-change of the acid Bohr groups on oxygenation is the same as in unsubstituted hemoglobin. Curve D in Fig.3, which shows the residual Bohr effect after substitution, supports this conclusion. It is seen that the alkaline Bohr effect is reduced to about half the original value and that the acid Bohr effect is hardly changed. Since the acid Bohr groups are not affected by substitution, it seems likely that the conformational change of Hb at oxygenation, which is believed to be responsible for the Bohr effect, is likewise

not affected*. So the conclusion seems justified that two of the four normal Bohr groups have been blocked. If this is correct, the third blocked group is probably an imidazole group. In view of the results already mentioned of Hill and Davis[11] and of Neer and Koningsberg[25], who found that the α-amino groups of the α-chains react predominantly over other groups, the α-amino groups of the β-chains included, it seems allowed to conclude that the α-amino groups of the α-chains are two of the normal Bohr groups. Calculated curves, based on the assumptions discussed above, are given in Fig.5 (curves C and D) and in Fig.3 (curve E). The agreement with the experimental curves is quite reasonable, especially in those parts of the curved where the acid Bohr groups do not contribute. It is possible that the pK of the acid Bohr groups differ somewhat from the values that were chosen. Moreover, the assumption that all four acid Bohr groups have the same pK is possibly not correct. Nevertheless, the calculated curves support our conclusion about the alkaline Bohr groups. There is, however, one point which is in contrast with the results of other authors. While in our case still fifty percent of the alkaline Bohr effect is present, Neer and Koningsberg[25] report that, on blocking the α-amino groups of the α-chains in human hemoglobin, the Bohr effect has been disappeared completely. On the other hand, Antonini (cited in ref.10, footnote 8) says "that some hemoglobins, which lack free α-amino groups, may still have a nearly normal Bohr effect". Also Zito and Brunori - cited in ref. 26 - have found that when the α-amino groups of hemoglobin are converted to a very weak or nonionizable form the Bohr effect remains unchanged.

To obtain more information we have studied the titration curves of hemoglobin in which more groups are substituted by DNP. The substitution reaction was again performed at 25^{o} at pH = -7.2, but now in a saturated solution of FDNB, during 2 hours; the unreacted FDNB was removed by dialysis at 4^{o}. The results are given in Fig.6. The number of groups that have reacted is 6.5; 5 from the neutral region, for the mean distance between the left and the right peaks is 16.8, and 1.5 groups

* Recently Dr. J.V. Kilmartin and Dr. L. Rossi-Bernardi have informed us that they also have found evidence that, when the α-amino groups of the α chains of horse hemoglobin are specifically modified by reaction with cyanate, the conformational change at oxygenation is not affected.

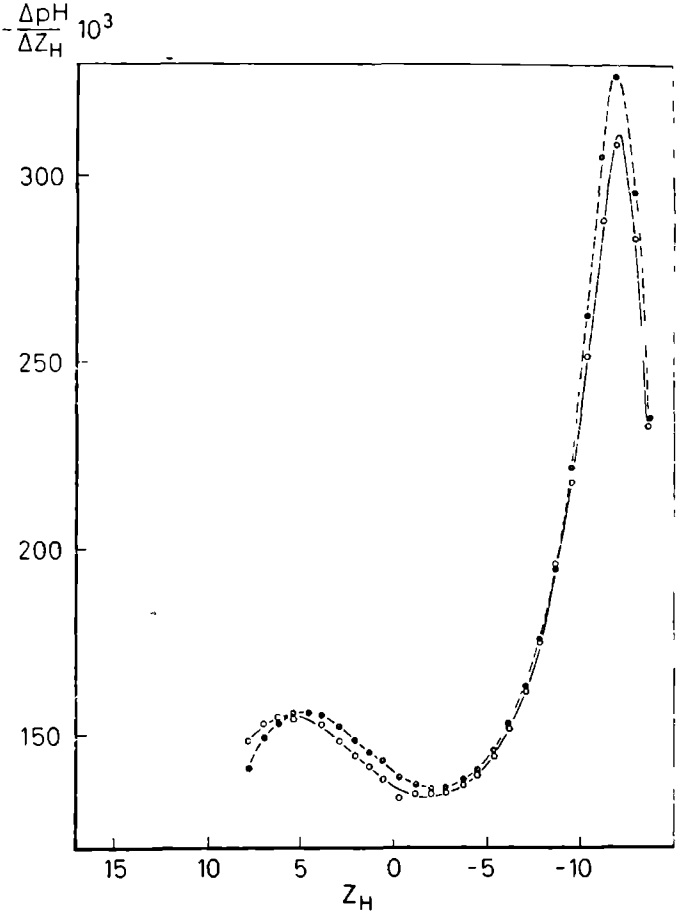

Fig.6. Differential titration curves of oxygenated (o) and deoxygenated (•) DNP-substituted hemoglobin at 25°.
The reaction was carried out in a satd. soln. of FDNB during 2 h at 20° and pH = 7.2.

from the basic region because the position of the right peaks is shifted to $Z_H = -11.8$. It is seen that not only the normal Bohr effect is now absent, but also the acid Bohr effect. We think that it is therefore not allowed to conclude that the remaining two alkaline Bohr groups also have been blocked. It is also possible that the many groups, which have reacted, now prevent the conformational change at oxygenation. The small difference in peak position of the two left peaks is possibly caused by the decreased stability of the substituted hemoglobin at the acid side of the titration curve.

The problem of the identity of the two other alkaline Bohr groups still remains. In this respect the observations of Taylor et al.[27] may be of interest. They found a similar decrease of the alkaline Bohr effect if the two SH-groups at position β 93 in human hemoglobin - in bovine hemoglobin at position β 92[14,15] - were blocked, e.g. by cystamine. Since Neer and Koningsberg[25] have found that these SH-groups are not blocked by FDNB, it is tempting to suppose that these groups are the other normal Bohr groups. Unfortunately Taylor et al. do not indicate the pK-value of these groups, while Guidotti[28] states, assuming that cystamine reacts with the ionized state of the SH groups, that their pK may be larger than 9 5. This would rule out the possible role of these groups as Bohr groups Differential titration curves after combined blocking with FDNB and cystamine will possible give an answer to this question. Because, if reaction with cystamine will reduce the Bohr effect without altering the distance between the two peaks in Hb and HbO_2, they cannot be the other normal Bohr groups

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Chapter 4

CHARGE FLUCTUATIONS IN ISOIONIC PROTEIN SOLUTIONS

4.1. Introduction

A protein molecule has many groups able to exchange protons with the solvent. From the theory, outlined in chapter 1, it follows that the number of bound protons for each individual macroion fluctuates around a mean value $\bar{\nu}$. So at any pH molecules will be present having a number of bound protons equal to $\bar{\nu}, \bar{\nu} \pm 1$, etc. A measure for the distribution of ν around the mean value $\bar{\nu}$ is the standard deviation S, represented by - see chapter 1 -

$$S^2 = \overline{\nu^2} - \bar{\nu}^2 \qquad (4.1.1)$$

S can be expressed as a function of the proton charge Z of the several ionic species*. Z is related to the number of protons bound by the eqn.

$$Z = \nu - \bar{\nu}_{iso} \qquad (4.1.2)$$

in which $\bar{\nu}_{iso}$ is the average number of protons bound at the isoelectric point. Combining eqns. (4.1.1) and (4.1.2) we can write

$$S^2 = \overline{Z^2} - \bar{Z}^2 \qquad (4.1.3)$$

Especially in the case of salt-free isoionic protein solutions the quantity S is important. Under these conditions ($\bar{Z} = 0$) S^2 is equal to $\overline{Z^2}$. It can easily be shown, by applying the Debye-Hückel theory[1], that the contribution of the charge fluctuations to the chemical potential of the protein in an isoionic solution is negative and is determined by the value

* Note:
In chapter 2 and 3 Z_H stands for the mean proton charge of the protein. In this chapter this quantity will be indicated by $\bar{Z}$ and Z will stand for the actual number of protons bound at a certain time.

of S. In a more general treatment - also for $\bar{Z}$ values different from $\bar{Z}$ = 0 - Kirkwood and Shumaker[2] have calculated the influence of the charge fluctuations on the chemical potential of the protein solution. In the case of isoionic salt-free solutions nearly the same expression is obtained as by applying the Debye-Hückel theory. This negative contribution and so S can be measured by light scattering techniques, as has been performed by Timasheff et al.[3-5].

Linderstrøm-Lang (see chapter 1) has shown that S^2 is also directly proportional to the slope of the hydrogen ion titration curve; so S can determined from this slope. Van Os, Möller and Overbeek[6-8] have investigated the conductance of alkali albuminates as a function of $\bar{Z}$. They found that at Z values near zero the equivalent conductance of the alkali ions exceed their limiting values. They explained this by assuming a contribution of charge fluctuations to the conductivity of the protein solution, and pointed out that it must be possible to determine S at $\bar{Z}$ = 0 from conductance measurements. In this chapter we present a quantitative measurement of the conductivity of isoionic protein solutions, due to charge fluctuations only. A relationship will be given between this conductivity and the quantity S. The results will be compared with values of S obtained from the slope of hydrogen ion titration curves at $\bar{Z}$ = 0. The proteins investigated were bovine carbon monoxide hemoglobin (HbCO), horse heart metmyoglobin (MetMb), bovine methemoglobin (MetHb) and bovine serum albumin (BSA).

4.2. Methods and Materials

A precision conductivity bridge, which has been decribed previously[9], was used for conductivity measurements. All measurements were carried out at $25^{o} \pm 0.02$ at a frequency of 1000 c.p.s. Because the conductivities of isoionic protein solutions are very low, care had to be taken that the solutions were completely salt-free during measurement. For example, electrolyte given off by the glass walls of the conductivity cell strongly interferes with the measurements. Materials other than glass did not give satisfactory results, either because of insufficient stability of the cell constant (plastic cells) or for difficulties in obtaining a tight joint between platinum and the cell material (plastic, quartz). We therefore used a modified low conductivity Jones cell[10,11],

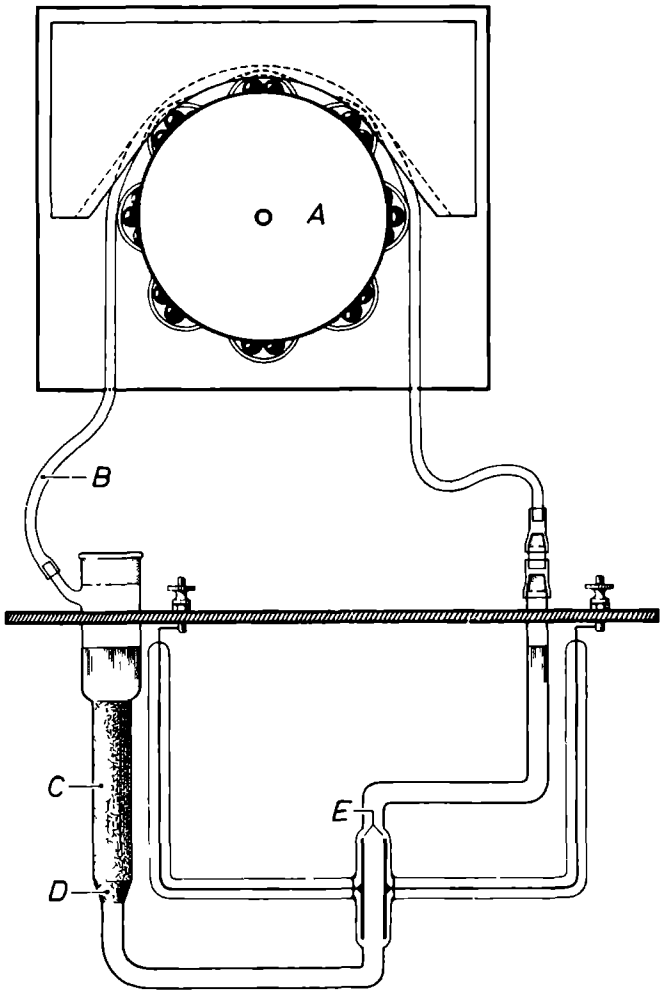

Fig.1. Modified low conductivity Jones cell.

A: peristaltic pump.

B: PVC-tubing (Tygon, LKB-Produkter, Sweden); length 25 cm; i.d. 4 mm; o.d. 5.5 mm.

C: ion exchange column.

D: quartz wool.

E: Pt-electrodes.

The cell constant was 0.03830 cm

as shown in Fig.1. One leg of the cell, which was made of Jena 16^{III} glass, was replaced by a small column filled with a mixed bed of ion-exchange resins (Amberlite IRA 400 and IR 120).The contents of the cell, including the column, amounts to about 15 ml. The liquid was pumped around by means of a peristaltic pump. With a flow rate of 20 ml/min the theoretical conductance of water ($5.4 \times 10^{-8}\ \Omega^{-1}cm^{-1}$) was reached to 1°/o within about 10 minutes, after the cell was filled with water containing some KCl. The use of quartz wool instead of glass wool, at the bottom of the column, appeared to be essential. When glass wool or sintered glass was used this theoretical value could not be reached. Measurements on proteins were carried out by withdrawing about 3 ml water from the system and replacing it by equal volume of a previously already deionised protein solution. Constant resistance readings were then always obtained within 5 minutes. The concentration of the protein solution was determined afterwards spectrophotometrically, using the following extinction coefficients (1 cm, 1°/o): MetHb: E = 5.21 at 500 mμ ; HbCO: E = 8.40 at 540 mμ ; MetMb: E = 3.42 at 505 mμ ; BSA: E = 6.68 at 278 mμ . The slope of the hydrogen ion titration curve was determined as described in chapter 2.

The isoionic pH of a protein solution was measured using a glass electrode and a calomel electrode with a very small KCl-leak. The solution was held in the deionised state by pumping it continuously through a small ion exchange column in a way similar to that described above. In these salt free solutions the unknown liquid junction potential may cause some error in the isoionic pH.

Bovine Hb was prepared by the method of Drabkin[12] and converted to HbCO or MetHb. MetMb and BSA were purchased from the Nutritional Biochemicals Corporation.

4.3. Results and Discussion

A. Conductance measurements

Due to the charge fluctuations an isoionic protein solution can be regarded as a solution containing a mixture of positive and negative univalent ions, bivalent ions, trivalent ions, etc. The macroions act as counterions for each other in the same way as in solutions of strong electrolytes. For this mixture of macroions wo have the following ex-

pression for the specific conductance $x_p(\Omega^{-1}\ cm^{-1})$ of the protein

$$1000\ x_p = \sum_i Z_i F v_i\ c_i \qquad (4.3.1)$$

where Z_i is the actual proton charge of the ion species i, v_i (cm . s^{-1}) its mobility, c_i its concentration (moles/litre) and F the Faraday (Coulombs). Making the reasonable assumption that at a certain protein concentration v_i will be proportional to Z_i, as long as Z_i is small, we may write

$$v_i = b\ Z_i \qquad (4.3.2)$$

The value of b depends upon the frictional coefficient of the protein and it will be dependent on concentration because of the mutual interactions between the macroions. Substituting equation (4.3.2) into (4.3.1) and dividing by the total concentration $c = \sum_i c_i$ eqn.(4.3.1) becomes:

$$\frac{1000\ x_p}{c} = bF\ \frac{\Sigma c_i\ Z_i^2}{\Sigma c_i} = bF\overline{Z^2} \qquad (4.3.3)$$

If c is given in grams per 100 ml (practically equal to grams per 100 g solution) eqn. (4.3.3) becomes:

$$\frac{1000\ x_p}{c} = \frac{10bF}{M}\ \overline{Z^2} \qquad (4.3.4)$$

where M is the molecular weight.

To obtain x_p we have to subtract from the total specific conductance of the solution the contribution of the hydrogen and hydroxyl ions, equal to $\lambda_{H^+} C_{H^+} + \lambda_{OH^-} C_{OH^-}$ (λ = equivalent conductance). In the case of MetMb, HbCO and MetHb, isoionic points were found near of pH = 7.3 (Table I). To calculate the contributions of the H^+ and OH^- ions to the conductance, two assumptions were made. Firstly the concentrations were calculated from the relation pH = $-\log c_{H^+}$, i.e. an activity coefficient of 1 was assumed. Secondly the limiting values for λ_{H^+} and λ_{OH^-} were used. These assumptions are allowable in these cases, because x_p is much greater than the contribu-

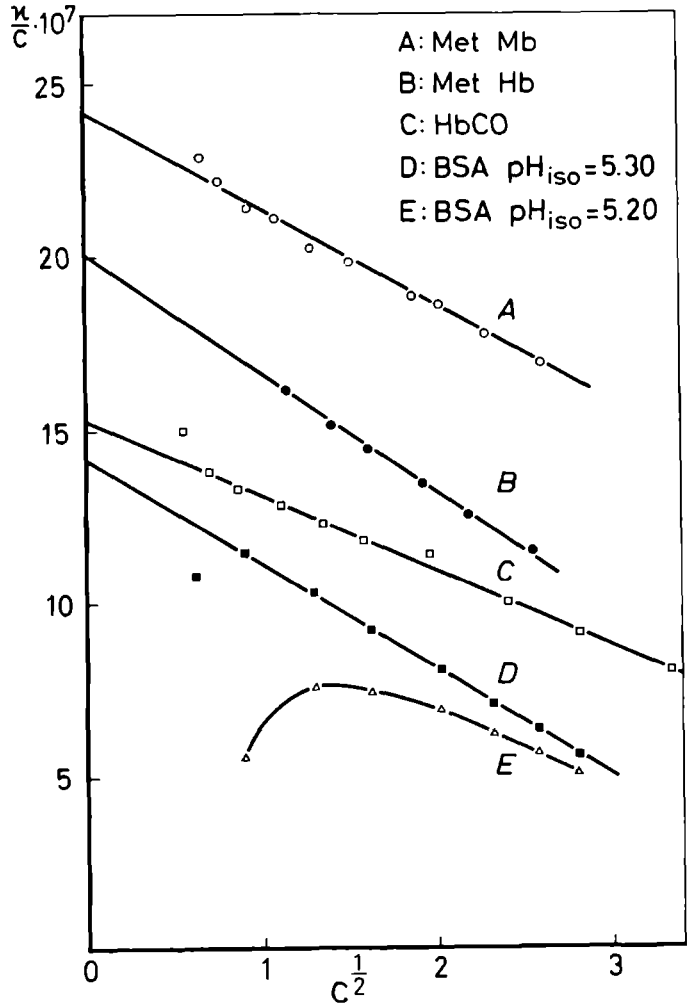

Fig.2. x_p/c as a function of c (c in weight per cent).
Curve A: MetMb; Curve B: MetHb; Curve C: HbCO;
Curve D: BSA, assuming pH_{iso} = 5.30;
Curve E: BSA, assuming pH_{iso} = 5.20.

tion of the H^+ and OH^- ions, when the protein concentration is not too low. Moreover, the errors resulting from the two assumptions tend to compensate each other, since c_{H+} will be higher than a_{H+}, while λ_{H+} and λ_{OH-} will be smaller than their limiting values. In Fig.2 x_p/c is given as a function of $c^{\frac{1}{2}}$ (c = protein concentration in weight percent) for MetMb, MetHb and HbCO (curves A,B and C). It is seen that x_p/c shows an Onsager concentration dependence. The linear relationship between x_p/c and $c^{\frac{1}{2}}$ strongly supports the supposition[1] that the extra free energy of the isoionic protein solution as a result of the charge fluctuations can indeed be calculated by applying the Debye-Hückel model of electrolytic solutions.

Table I

S_{cond} for the four proteins investigated, as calculated from the limiting conductance (x_p/c at c = 0), using the molecular weight M, the molecular radius R and the isoionic point pH_{iso}.

	M	R(Å)	pH_{iso}	$(x_p/c).10^7$ at c = 0	S_{cond}
MetMb	17.800	18.4	7.36	24.2	0.984
MetHb	65.000	29.0	7.36	20.1	2.15
HbCO	65.000	29.0	7.25	15.3	1.88
BSA	69.000	30.0	5.30	14.2	1.89

The calculation of x_p for BSA is somewhat more complicated. For the isoionic pH of salt-free BSA we found 5.20; the contribution of the H^+ and OH^- ions to the conductance therefore became of the same order of magnitude as x_p. When c_{H+} was taken equal to a_{H+} and the limiting values of λ_{H+} and λ_{OH-} were used, curve E of Fig.2 was obtained. The course of this curve seems unlikely, for the BSA-molecule has about the same mass and frictional coefficient as the Hb molecule. Since it is not probable that our two assumptions about the H^+ and

OH^- ions can cause large errors, we think that the isoionic point of 5.20 is not quite correct. This is possible because pH-measurements in salt-free protein solutions may be influenced by the unknown liquid junction potential. When an isoionic point of 5.30 was chosen, curve D of Fig.2 was obtained.

From eqn. (4.3.4) Z^2 can now be derived if the constant b is known. If the macroions are spherical, Stokes' law will hold at infinite dilution and b* will then be given by[1]:

$$b = \frac{1}{300} \cdot \frac{e}{6\pi\eta R} \qquad (4.3.5)$$

in which e is the elementary charge (= 4.8 . 10^{-10} e.s.u.), η the viscosity of water at 25° (= 8.94 . 10^{-3} poises) and R is the equivalent sphere radius of the protein. The latter is defined[1] as the radius of a sphere with a volume equal to that of the protein molecule. Eqn. (4.3.4) then becomes (4.3.6)

$$\frac{x_p}{c} = \frac{10^{-4}}{3} \cdot \frac{eF}{6\pi\eta} \cdot \frac{\overline{Z^2}}{MR} = 8.2 \cdot 10^{-9} \cdot \frac{\overline{Z^2}}{MR}$$

R was calculated[1] from the eqn. $\frac{4}{3}\pi R^3 = \frac{M}{N}(\bar{v}_2 + \delta_1 v_1^o)$ in which N is Avogrado's number, v_1^o the pure solvent specific volume, $\bar{v}_2$ the partial specific volume of the protein and δ_1 the amount of the solvent in grams bound per gram of dry protein. The values of $\bar{v}_2$ and δ_1 were taken from the literature (see ref.1, p.358,359 and 467). Since eqn. (4.3.6) is valid only at infinite dilution, values of x_p/c at c = o have to be used in the calculation of $\overline{Z^2}$. These were found by extrapolating the curves in Fig.2 to c = o. Table I gives the results for S_{cond}, together with some other parameters of the protein molecules.

* Note:
The same expression for b is obtained by applying the more general approximate Booth eqn. - ref.1 -, for at infinite dilution the Debye-Hückel parameter is equal to zero.

B. Hydrogen ion titrations

According to Lindstrøm-Lang the quantity $S^2 = \overline{Z^2} - \bar{Z}^2$ is directly related to the slope of the hydrogen ion titration curve at the point $\bar{Z}$ by the eqn.

$$\frac{d\bar{Z}}{dpH} = -2.303(\overline{Z^2} - \bar{Z}^2) \qquad (4.3.7)$$

At the isoionic point $\bar{Z}$ is in practice equal to zero and so eqn. (4.3.7) becomes

$$\left(\frac{d\bar{Z}}{dpH}\right)_{\bar{Z}\,=\,o} = -2.303\ \overline{Z^2} \qquad (4.3.8)$$

So $\overline{Z^2}$ can be found by measuring $d\bar{Z}/dpH$ at the isoionic point. However, a comparison of this value with that found from conductivity measurements requires that $d\bar{Z}/dpH$ must be measured under the same conditions used in deriving the conductance values, i.e. for a salt-free protein solution at the limit of zero protein concentration.The titration curve of a protein, however, can only be determined at a finite ionic strength and protein concentration . So the problem is how to find $d\bar{Z}/dpH$ at $I = o$ and $c = o$. We have tried to solve this problem in the following way. The slope of the titration curve of a protein at point $\bar{Z}$ can also be expressed in the molecular parameters describing the proton exchange behaviour of the protein with the solvent, giving the relation (see chapter 2) (4.3.9)

$$-\frac{dpH}{d\bar{Z}} = +\ 0.868\ w + \frac{1}{2.303 \sum_i n_i\ \alpha_i(1-\alpha_i)}$$

with

$$\bar{Z} = Z_m - \sum_i \alpha_i\ n_i \qquad (4.3.10)$$

For a compact spherical molecule w is given by

$$w = \frac{e^2}{2DRkT}\left(1 - \frac{xR}{1 + xa}\right) \qquad (4.3.11)$$

Eqn. (4.3.11) is valid only if the macroions "do not see each other", i.e. if the double layers do not overlap. This is true: a. at finite protein concentration when 1/x is small enough, thus when the ionic strength is high enough. b. in salt-free protein solutions at the limit of zero protein concentration. In this case eqn. (4.3.11) reduces to $w = e^2/2\,DRkT$.

Using eqns. (4.3.9) and (4.3.10) $dpH/d\bar{Z}$ at $I = o$ and $c = o$ can now be derived in the following way. First dpH/dZ is determined experimentally at sufficiently high ionic strength and finite protein concentration. With I known w is calculated according to eqn. (4.3.11). The second term (y) on the right-hand side of eqn. (4.3.9) can now be calculated. This term is independent of the protein concentration and thus y retains its value at $c = o$. Now w is calculated at $I = o$ and $c = o$, by setting $x = o$. Substitution of this w together with y in eqn. (4.3.9) then yields dpH/dZ at $I = o$ and $c = o$. For example the differential titration curve of HbCO - which appeared to be identical with that of HbO_2[13] - at $I = 0.066$ and $c = 1^o/o$ gives $dpH/d\bar{Z} = -0.110$ at $\bar{Z} = o$. Eqn. (4.3.11) yields at $I = 0.066$ $w = 0.041$ giving according to eqn. (4.3.9) $y = -0.074$. At $I = o$ $(x = o)$ eqn. (4.3.11) gives $w = 0.123$, so $dpH/d\bar{Z}$ becomes $-0.107 - 0.074 = -0.181$. From eqn. (4.3.8) $Z^2 = 2.40$ is so obtained.In Table II the results are listed for HbCO and the other proteins while in Table III the main results are summarized. The agreement between the two values for S is quite reasonable, especially in view of the fact that the two ways of measuring S are completely independent and that several assumptions had to be made. Nevertheless, it is striking that there is a systematic deviation between S_{cond} and S_{titr}. The latter is found always smaller than S_{cond}. It will therefore be of interest to discuss the consequences of the most important assumptions. First we assumed that w is given by eqn. (4.3.11). The derivation of this eqn. is based upon the assumption that the molecule can be approximated by a sphere with its charge evenly distributed over the surface the macroion. However, this assumption is a poor approximation. For the proteins investigated w is mostly found experimentally to be about $20^o/o$ smaller than the value calculated from eqn. (4.3.11)[14-16].

We have even found - chapter 3 - a value for w being about $50^o/o$ smaller than the calculated value. Since, as can be seen in Table II, w has a large influence on the value of $dpH/d\bar{Z}$ at $I = o$, it seems likely that the difference between S_{cond} and $S_{titr.}$ is caused chiefly by too large values of w being used in the calculation of S_{titr}.

Table II

S_{titr} for the four proteins investigated, as calculated from the slope of the titration curve (-dpH/dZ) at the ionic strength I. See text for the other parameters. For the difference between a and R the usual value of 2.5 Å was chosen (see for example ref.14).

	I	R(Å)	a(Å)	w	$w_{I=0}$	$-\frac{dpH}{dZ}$	-y	$\left(-\frac{dpH}{dZ}\right)_{I=0}$	S_{titr}
MetMb	0.025	18.4	20.9	0.105	0.193	0.490	0.399	0.567	0.875
MetHb	0.060	29.0	31.5	0.042	0.123	0.112	0.076	0.183	1.54
HbCO	0.066	29.0	31.5	0.041	0.123	0.110	0.074	0.181	1.55
BSA	0.062	30.0	32.5	0.039	0.119	0.105	0.071	0.174	1.58

Table III

Comparison of S_{cond} and S_{titr}.

	S_{cond}	S_{titr}	S_{cond}/S_{titr}
MetMb	0.984	0.875	1.12
MetHb	2.15	1.54	1.39
HbCO	1.88	1.55	1.21
BSA	1.89	1.58	1.20

Another important assumption, used in the calculation of S_{cond}, is that the frictional coefficient is given by $6 \pi \eta R$, i.e. that the molecules are spherical. For the heme proteins investigated this cannot lead to appreciable errors, since these molecules are all nearly spherical and have in solution a Perrin factor* near 1.0[17]. The BSA molecule, however, is not spherical. Bloomfield[18], Peters and Hawn[19] and Squire et al.[20] have found strong indications that the shape of the molecule can be represented by a prolate elipsoid with an axial ratio of about 3, leading to a Perrin factor of about 1.2. Consequently, $\overline{Z^2}$ increases about 20°/o, but S_{cond} accordingly only about 4°/o.

It should be noted further that in deriving eqn. (4.3.6) we have made the implicit assumption that a molecule immediately attains the velocity belonging to the new charge, or in other words that the lifetime of a molecule in a charged state Z is long compared with the time needed to reach its uniform velocity[6]. When Stokes' law holds, the time constant τ of this accleration period is given by[6]

$$\tau = \frac{m}{6 \pi \eta R} \qquad (m = \text{molecular mass})$$

For the proteins investigated τ is of the order of 10^{-12} s. Weak electrolytes, like acetic acid, have a time constant of about 10^{-14} s and because the dissociation constant obtained from conductance measurements and from the hydrogen ion titration curve of acetic acid solutions are the same, the condition mentioned above seems to be fulfilled. Although this will also be roughly valid for the individual groups of the protein, it is a priori difficult to say whether the condition is also satisfied for the protein itself because the charge Z of the protein is made up of many ionisations. However, if this were not so, S_{cond} would become smaller and in view of the reasonable agreement between S_{cond} and S_{titr} it seems to be justified to conclude that for proteins also the time constant is small with respect to the lifetime of the protein in the charged state Z.

* Note:
Perrin's factor is defined as $\frac{f}{f_o}$, in which $f_o = 6 \pi \eta R$ and f the real frictional coefficient. This factor is always larger than 1.0 for particles having a shape which can be represented by a prolate or oblate elipsoid.

It may be mentioned that Timasheff et al.[5] have found for BSA by light scattering techniques S = 3.58. From the slope of titration curve of BSA, as given by Tanford et al.[14], they calculate S_{titr} = 3.46, in very good agreement with the light scattering value. There must, however, be some mistake in this calculation since we found from the slope of Tanford's titration curve a value for S_{titr} of about 1.6.This value is in good agreement with our S_{titr} (1.58), but not with the light scattering value. Moreover, an estimation of S_{titr} from an analysis of the titration curve (numbers of groups in each class with reasonable values for the pK_i's and for w) also leads to a value of about 1.6[14] - see also chapter 2 -. We have no explanation for the discrepancy between the light scattering value and the values presented by us. Some light scattering data for the hemeproteins are available[21,22] but as far as we know not for salt-free isoionic solutions, so that for these proteins a comparison with S_{cond} and S_{titr} is not possible.

Recently Baskin[23] has made electric conductance measurements with isoionic salt-free bovine mercaptalbumin as a function of the protein concentration. From his results he calculated the hydrogen ion concentration and the net charge of the protein. In these calculations he assumed that the protein does not contribute to the conductance of the solution. In this way he obtained S = 1.03 which is remarkably lower than the value reported by us - Table I -. We corrected, however, his data as good as possible for x_p and found S = 1.35 which is in better agreement with our value.

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Samenvatting.

De onderwerpen, die in dit proefschrift aan de orde komen, omvatten studies van het proton uitwisselingsgedrag van eiwitten.
In hoofdstuk 1 wordt een kort overzicht gegeven van de theorie der multipele evenwichten. De statistische behandeling van dit onderwerp verschilt enigszins van de gebruikelijke wijze van benadering. Met name zijn van het begin af aan activiteitscoëfficiënten in de theorie opgenomen. Dit kort overzicht is opgenomen, daar beide onderwerpen van onderzoek zoals gezegd een studie zijn van het evenwicht tussen protonen in oplossing en de talrijke bindingsplaatsen aan het oppervlak van een eiwitmolecuul.
In hoofdstuk 2 wordt een verfijning beschreven van de gebruikelijke titratie procedure. Deze verfijning maakt het mogelijk om nauwkeurig de differentiële titratiecurven van eiwitten te bepalen. Het voordeel van deze curven is dat zij direct meer kwalitatieve informatie geven dan de normale titratiecurven. Tevens wordt een theoretische analyse procedure voorgesteld met behulp waarvan de experimentele curve nader kan worden geïnterpreteerd. De mogelijkheden van de methode worden in dit hoofdstuk geïllustreerd aan de hand van de resultaten verkregen voor runder serum albumine. De voornaamste hiervan kunnen als volgt worden samengevat:

Het aantal titreerbare carboxylgroepen overtreft de maximale positieve protonlading van het eiwit. Het verschil bedraagt ongeveer 5. Uit de vorm van de differentiële titratiecurve blijkt dat er waarschijnlijk een conformatie overgang plaats vindt in het pH gebied tussen 7 en 9, welke de pK van de imidazol groepen beïnvloedt. Uit een vergelijking van de differentiële titratiecurven van gedialyseerd en gedeioniseerd albumine blijkt dat aan gedialyseerd albumine enkele vetzuurmoleculen zijn gebonden, die door deionisatie via een gemengde ionenwisselaar verwijderd kunnen worden.

Hoofdstuk 3 handelt over het Bohr effect van runder hemoglobine en van runder hemoglobine na reactie met 1-fluoro-2.4-dinitrobenzeen.
Dit effect werd bestudeerd aan de hand van normale, differentiële en verschil titratiecurven. De belangrijkste resultaten zijn de volgende:
De aanwezigheid van de alkalische en zure Bohr groepen volgt direct uit een onderlinge vergelijking van de differentiële titratiecurven van gereduceerd en geoxygeneerd hemoglobine.
Een vergelijking van de differentiële en verschiltitratiecurven van ongesubstitueerd en gesubstitueerd hemoglobine leidt tot de conclusie dat 2 van de 4

α-amino groepen deel uit maken van de alkalische Bohr groepen.
Een analyse van de differentiële titratiekrommen van ongesubstitueerd hemoglobine toont aan dat slechts 18 van de 32 aanwezige histidine groepen titreerbaar zijn, en dat 4 van deze 18 een abnormaal hoge pK van ongeveer 8 hebben.
Hoofdstuk 4 beschrijft een onderzoek naar de ladingsfluctuaties van eiwitten in isoionische zoutvrije eiwitoplossingen. Dit effect werd bestudeerd gebruik makend van conductometrische en titrimetrische analysemethoden.
De geringe bijdrage van het eiwit tot het geleidingsvermogen van een isoionische eiwitoplossing, veroorzaakt door ladingsfluctuaties, kon gemeten worden door de eiwitoplossing herhaaldelijk te leiden over een kolom gevuld met gemengde ionenwisselaar, waarbij gebruik werd gemaakt van een rondpompsysteem. Op deze wijze worden bijdragen van vreemde ionen tot het geleidingsvermogen volkomen geëlimineerd. Via deze werkwijze kan het theoretisch geleidingsvermogen van water ($5.4 \times 10^{-8}, \Omega^{-1}\ cm^{-1}$) snel worden bereikt.
Voor het specifiek geleidingsvermogen van de onderzochte eiwitten per eenheid van concentratie werd een Onsager afhankelijkheid aangetoond. Gebruik makend van enige redelijke benaderingen werd informatie verkregen over de standaarddeviatie S van de ladingsfluctuaties. De verkregen resultaten stemmen vrij goed overeen met de waarden voor S berekend uit de helling van de titratiekromme in het isoionisch punt.

Summary

This thesis deals with the proton exchange behaviour of proteins. The mean features can be summarized as follows.

In chapter 1 a short survey is given of the theory of multiple equilibria. The statistical treatment of this subject differs somewhat from the usual treatment, especially with respect to the correction factor for the electrostatic interactions. This treatment has been inserted in this thesis, because the areas of investigation presented, are studies of the equilibria between the hydrogen ions in solution and the numerous proton binding sites on the protein molecule.

In chapter 2 a refinement is described of the usual titration procedure which has enabled us to measure differential titration curves of proteins with good precision. In addition a quantitative theoretical analysis of these curves is given.

The capabilities of the method are demonstrated in this chapter by showing the results obtained for bovine serum albumin. These can be summarized as follows:

The number of titratable carboxyl groups exceeds the maximum positive proton charge. The difference is about 5 groups.

From the shape of the differential titration curve it appears that probably a conformational change takes place in the pH-region between 7 and 9, thereby influencing the pK of the imidazole groups.

A comparison of the differential titration curves of dialysed and deionised albumin shows that some fatty acid molecules are bound by dialysed albumin, which can be removed by deionisation, using a mixed bed ion-exchange column.

Chapter 3 deals with the Bohr effect of bovine hemoglobin and of bovine hemoglobin after reaction with 1-fluoro-2.4-dinitrobenzeen.

This effect has been studied by means of normal, differential and difference titration curves. The study reveals the following facts:

The presence of the normal and acid Bohr groups is easily seen from the differential titration curves of deoxygenated and oxygenated hemoglobin respectively.

An analysis of these curves shows that only 18 of the 32 histidine residues are titratable and that 4 of these 18 residues have an abnormally high pK of about 8 in deoxygenated as well as in oxygenated hemoglobin.

From a comparison of the differential and difference titration curves of

DNP-substituted hemoglobin with those of unsubstituted hemoglobin it is concluded that 2 of the 4 α -amino groups are alkaline Bohr groups.

Chapter 4 presents a study of the charge fluctuations of proteins in isoionic saltfree solutions. The standard deviation S of these charge fluctuations has been studied by means of conductivity measurements and hydrogen ion titrations.

The small contribution of proteins to the conductance of isoionic protein solutions, due to charge fluctuations, could be measured by passing the protein solution repeatedly over a mixed bed of ion-exchange resin using a recycling system. In this way the contribution to the conductance of interfering electrolyte could fully be eliminated.

By means of this recycling system we have reached the theoretical conductance of water ($5.4 \times 10^{-8}\ \Omega^{-1}\ cm^{-1}$). The specific conductance of the proteins per unit of concentration shows an Onsager dependence.

By making some reasonable assumptions S values for the several proteins at the limit of zero concentration could be calculated. The results so obtained agree fairly well with the S values calculated from the slope of the titration curves in the isoionic point.

STELLINGEN

I

De bewering van Hurd en Thomas, dat de vorming van dibenzylketon uit phenylazijnzuur en azijnzuuranhydride verloopt via het intermediair phenylazijnzuuranhydride, is niet juist.

C.D. Hurd and C.L. Thomas.
J. Am. Chem., Soc., 58 (1936) 1240.

II

De methode, die van Gool en Cleiren beschrijven om HCl-gas van O_2 verontreinigingen te zuiveren, is in zich niet mogelijk.

W. van Gool en A.P. Cleiren.
Z. Naturforschg. 16a(1961) 948.

III

De argumentatie, die Bielka en Lutsch tot de conclusie voert, dat Mg^{++} -ionen aan de basen van RNA van leverribosomen worden gebonden, is foutief.

H. Bielka and G. Lutsch.
Z. Naturforschg. 22b(1967) 1035.

IV

De door Irons en Perkins waargenomen toename in de binding aan albumine van Ca^{++} - en Sr^{++} -ionen bij verhoging van de metaalionenconcentratie is zeer onwaarschijnlijk; bovendien wordt deze door hen gevonden toename ten onrechte als elektrostatische binding geïnterpreteerd.

L.I. Irons and D.J. Perkins
Biochem. J., 84 (1962) 152.

V

Ten onrechte veronderstellen Bielka en medewerkers, dat uit hun sedimentatieexperimenten volgt, dat er een evenwicht bestaat tussen de 80S leverribosomen en hun subeenheden.

H. Bielka, H. Welfle, M. Böttcher en W. Förster
European J. Biochem., 5 (1968) 183.

VI

De statistische evaluatie procedure, zoals toegepast door Rossi-Bernardi en Roughton, ter analyse van de verschiltitratiekrommen van geoxygeneerd en gereduceerd hemoglobine, is aan bedenkingen onderhevig.

L. Rossi-Bernardi and F.J.W. Roughton.
J. Biol. Chem., 242 (1967) 784.

VII

Het nut van de telefoon op de lessenaar van de NOS-nieuwslezer is op zijn minst twijfelachtig.

25 september 1969.